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14. ABSTRACT

At the end of the 2nd year we have completed a >128 adult male and female mice for metabolomics, physiological responses, lipidomics and hormonal analysis at 6 time points following exertional heat stroke. The results demonstrate a greater resistance to exertional heat stroke in female vs. male mice, with a >1.5 fold increases in the capacity to perform work in the heat. Metabolomics in male mice demonstrated a conversion to higher beta-oxidation of fatty acids in the heart mitochondria. There were few other differences between male and female mice in terms of metabolism, but all exhibited an "energy crisis" and conversion to lipid and protein metabolism. Female mice had altered metabolic hormonal profiles during recovery with significant elevations in corticosterone, resistin, insulin and glucagon. Plasma immune system cytokines were generally elevated in female vs. male mice but had a similar pattern over time. Only IL-5 and IL-9 were present in females but not males. Mice given therapeutic levels of ibuprofen exhibited greater exercise performance in the heat but also greater small intestinal and gastric histological lesions

15. SUBJECT TERMS

Sex differences, exertional heat stroke, multi-organ injury, heat stress, metabolic hormones

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1. INTRODUCTION

Exertional heat stroke (EHS) is a serious medical problem in the U.S. Armed Forces, both during basic training and deployment operations. In the 2016 Medical Military Surveillance Report (1), there were 417 cases of heat stroke (largely EHS) and 2,350 cases of heat injury reported in the previous year. The rate of heat injury in active component members was 0.35/100 person years in males and 0.16/100 in females. The incidence rate of heat injury in males, however, were nearly identical. The reasons for these sex differences are not known. The Military needs solutions to determine when warfighters are fit to return to duty without further risk of EHS or other complications and whether there are long-term consequences of EHS that can be identified and treated. We have developed the first preclinical EHS model in mice that resembles the condition in humans. It is our aim to utilize this model to solve a series of problems related to EHS, to identify biomarkers that will translate to the conditions experienced by Warfighters, to evaluate the influence of common drugs and agents that may amplify the deleterious effects of EHS, and to develop treatment and prevention strategies that are applicable to the needs of military medicine. Ultimately, our goal is to save lives and suffering of US Military personnel.

There are four basic purposes of this project 1) To identify relevant biomarkers that could be helpful to the US Military in identifying effective and complete recovery from exertional heat stroke and in identifying risk factors for long-term complications of EHS. 2) Determine if there are significant differences in the response to EHS between males and females. 3) To determine if non-steroidal anti-inflammatory drugs (NSAIDs) impose additional risk factors for complications of EHS, and 4) To evaluate a new line NSAIDs that may offer a safe line of protection from organ injury in EHS. In the past two years we have completed 1-3 and are performing experiments for purpose 4 in the last year of the funding cycle.

2. KEYWORDS

Sex differences, exertional heat stroke, multi-organ injury, heat stress, metabolic hormones, non-steroidal anti-inflammatory drugs, biomarkers

3. ACCOMPLISHMENTS

What were the major goals of the project?

Year 1: 2 Months: Complete approval of IACUC protocols, coordinate the data collection schedule between 3 centers, set up of new equipment and attain approval of Environmental Risk Assessment.

6 Months: Study EHS in male mice: surgical implantation of transmitters, recovery, exercise training and collection of data from 56 mice exposed to EHS or exercise control. Mice will be studied in groups of 8, implanted 2 weeks apart.

2 Months: Submission of samples and analytical and morphological tests of organ and tissue injury, submission of samples for immunological studies, metabolic hormone studies, metabolomics and proteomics analyses and integration of data from 3 centers.

PROGRESS: All of year one goals were completed except for completion of the manuscripts, which are nearing submission.

Year 2: 6 Months: Study EHS in female mice: surgical implantation of transmitters, recovery, exercise training and collection of data from 56 female mice exposed to EHS or exercise control.
3 Months: Submission and analyses of samples for multiplex (Luminex) determination cytokines and metabolic hormones, development and testing of new assays for detection of targeted biomarkers from plasma and analyses of organ injury using histopathological analyses.

2 Months. Complete analysis and initial reports of metabolomics and proteomics, comparison of males and females and outcome of cytokine and metabolic hormone measurements.

PROGRESS: All studies planned for year two have been completed. Samples have been evaluated for metabolomics, metabolic hormone analysis and lipidomics. Data is still being analyzed and the first metabolomics manuscript is near completion.

Year 3: 4 Months: Completion of testing the impact of ibuprofen on organ injury in male and female mice during EHS in 48 mice. Submission of plasma samples for cytokine analyses and tissues for analysis of histopathological injury.

3 Months: Completion of testing for the impact of the predominant COX2 inhibitor, diclofenac vs. its H_2S-analog (ATB-337) on organ intestinal injury and damage to other organ systems following EHS in 32 male mice. Submission of plasma samples for cytokine analysis and multiple organ injury and for measurement of metabolomics and eicosanoid products.

3 Months: Completion of testing for the impact of the more predominant COX1 inhibitor, naproxen vs. its H_2S-analog (ATB-346) on organ intestinal injury and damage to other organ systems following EHS in 32 male mice. Submission of plasma samples for cytokine analysis and multiple organ injury and for measurement of metabolomics and eicosanoid products.

2 Months: Complete analysis of samples from mice, integrate data collection from the 3 laboratories and prepare final reports and manuscripts of experimental outcomes.

PROGRESS: The studies on males and females exposed to ibuprofen and EHA have been completed. We are currently doing an additional control series, analyzing histological samples and completing analyses.

What was accomplished under these goals? In the past year we completed the entire cohort of male and female mice exposed to EHS and collected samples at 6 time points up to 14 days of recovery. We also collected samples from controls. All samples have been analyzed in collaboration with our co-investigators at the USARIEM and USACEHR for metabolomics, lipidomics, metabolic hormone analyses and cytokines. We are completing a few additional analyses using targeted biomarkers and histological tissue analyses at UF, which are ongoing.

Highlighted findings:

Temperature profile to EHS in ♂ & ♀ mice
Females exhibited a significant resistance to EHS compared to age-matched male mice. Figure 1 shows a typical example of this phenomenon in two representative mice. The results are grouped in Fig 2 for all mice (N = 44 per group). As shown, female mice ran, on average, ~43% longer in the heat than male mice without losing consciousness. They were also exposed to a significantly higher overall heat loads because they ran longer in the heat (Fig. 2B, ascending thermal area). They exhibited a greater apparent fractional loss in body fluid, based on % body weight loss (Fig. 2C). Interestingly, both male and female mice collapsed, reaching the symptom limited end of EHS, at nearly identical peak core temperatures ($T_{c,max}$) and following EHS, their core temperatures dropped to nearly identical levels of hypothermia ($T_{c,min}$), Fig. 2D. This latter measurement is considered an index of severity of exposure to hyperthermic conditions (7). The specific results for the males in this study were very similar to results we published previously (6).

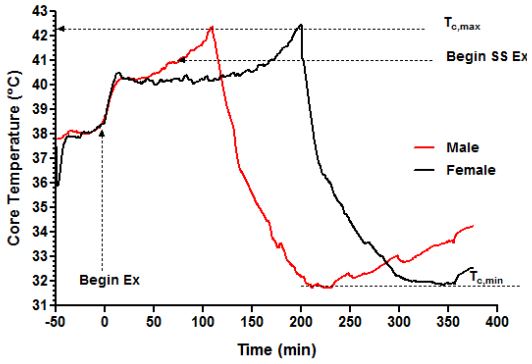


Fig. 1. Typical core temperature profiles of male (red and female mice during EHS trials. (in prep for publication)

Running Performance during EHS in ♂ & ♀ mice
Because the females could maintain their body temperature for longer periods during exercise in the heat, and the protocol called for incremental increases in running speed until a core temp. of 41°C was achieved, they ran at much faster speed (~39%) by the end of the EHS protocol (Fig. 3A) resulting in a total distance in the heat ~80% further than the comparable males (Fig. 3B).

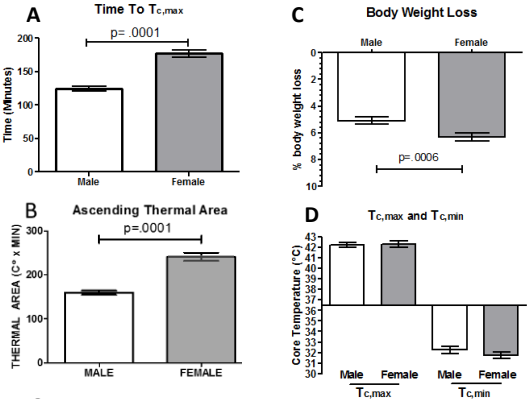


Fig. 2. Comparisons of ♂ & ♀mice of A) running time in the heat, B) thermal load, C) percent loss of body weight, and D) max and min core temperatures. Data are Means +/- SEM. (In prep for publication).

Causative factors for differences in heat tolerance and running performance during EHS between ♂ & ♀ mice.
We considered several "physical" factors in trying to explain the origins of the better heat tolerance and performance of the females compared to males. Males had significantly higher body weights (~14%) but females had significantly higher body surface area/mass ratios (and body surface to mass ratios, on average ~5% higher (Fig. 4). Therefore, presumably, females had a physical advantage due to the greater relative surface area for heat dissipation. The females also had a mechanical advantage because at a given speed they would be performing less mechanical work and therefore

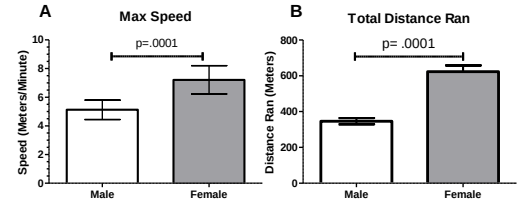


Fig. 3. Maximum speed achieved (A) and the total distance run in the heat in male and female mice. Means +/- SEM (in prep for publication)

producing less heat. The question we asked is whether we could account for the differences in performance between males and females in the heat, based only on differences in body surface area and power. The statistical results are shown in Figure 5. Body BSA/mass and Power output were good predictors of performance in both sexes but there remained a powerful effect of sex, particularly when expressed as a crossed effect with Power that could not be accounted for by power and BSA/m alone (Figure 5).

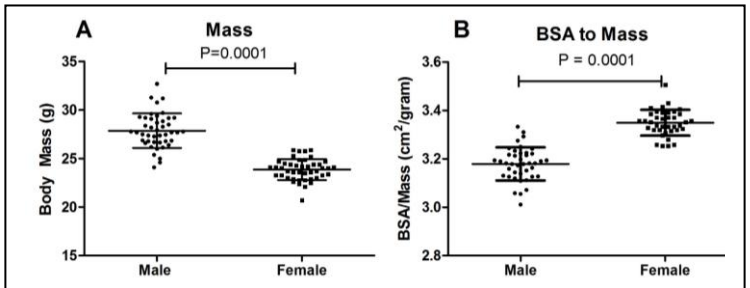


Fig. 4. Raw distributions showing **A)** The expected elevation in body mass of ♂ & ♀ mice, 20-24 weeks of age, and **B)** the calculated body surface area to mass ratio, showing that females had a greater surface area for heat dissipation (in prep for publication).

We took from this analysis, as well as other analyses of rates of heat dissipation (not shown), that independent of physical laws governing heat dissipation and external work, there was an independent influence of sex on heat tolerance and work capacity.

Differences in Metabolic regulation in ♂ & ♀ mice

In both males and females, there were statistically significant reductions in blood glucose as measured at 30 min and 3 hours but there were no differences between males and females except at the 9d time point. In general, glucose levels were higher than predicted in both groups between 1-14 days, as well as in the 4 d control animals (data not shown). *We conclude from this analysis that the mice go through a transient hypoglycemia that is resolved by 24 h post EHS. We do not believe that the hyperglycemia observed following EHS is due to EHS-induced pathology.*

We then measured metabolic hormone production in the plasma at each time point. The assays were run at USARIEM by Michelle King, Lisa Leon and colleagues. Corticosterone levels (the

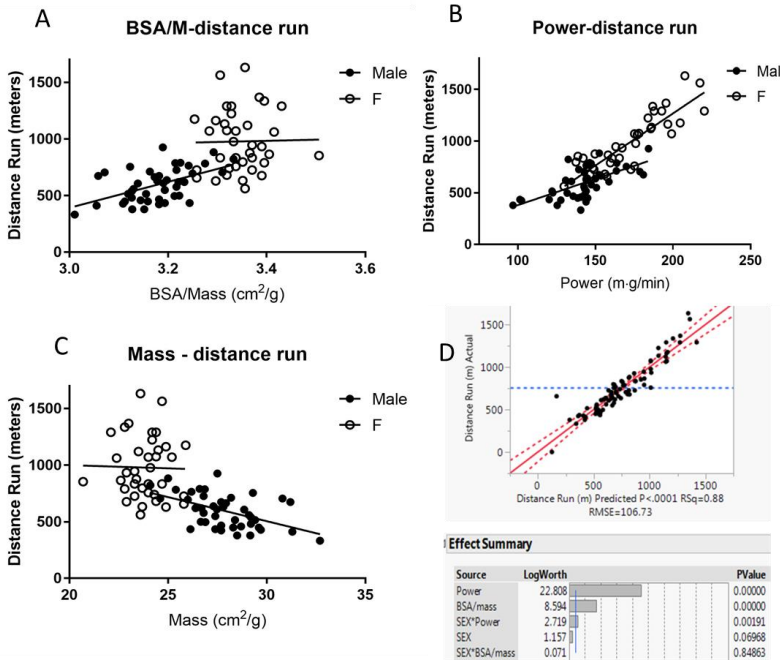


Fig. 5. Statistical modeling of physical determinants of differences in exercise performance in the heat between males and females. As predicted, both BSA/mass ratio (A) and Power (B) accounted for much of the variance in the distance run in the heat in both males and females. C) Mass is related to BSA/M ratio and demonstrates that weight appeared to be a more significant limiting factor for males. D) The total model for multiple linear regression using these variables. Sex as a significant predictor of outcome as a co-factor with power.

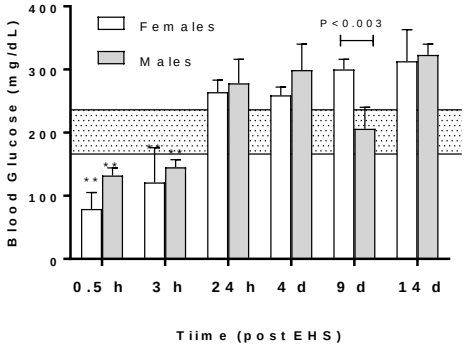


Figure 6. Blood glucose measurements at the time of sacrifice in male and female mice. Only at 9d was there any statistical differences in glucose.

primary glucocorticoid produced in mice) were highly elevated at the 30 min and 3h time points. Throughout recovery, females exhibited a higher corticosterone levels than males. At the 3 hr time point the corticosterone was nearly double in females compared to males. These data demonstrate that females have a more robust glucocorticoid response to EHS.

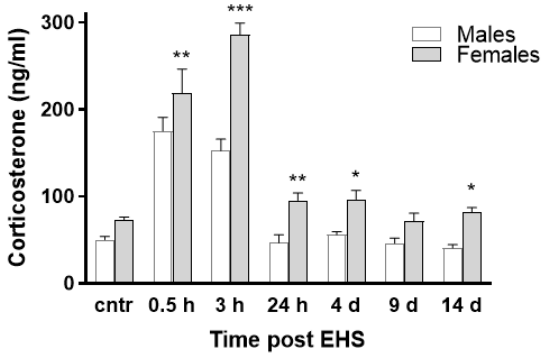


Fig. 7. Plasma corticosterone levels in sham control (cntrl) and during recovery from EHS. * P < 0.05 ** <0.01 differences between males and females at each time point.

Additional metabolic hormones were evaluated using Luminex multiplex technology (metabolic hormone panel). These results are displayed in figures 8 and 9. In figure 8, metabolic hormones arising from the pancreas are shown. Insulin and c-peptide (the latter a protein co-secreted with insulin from the β cells) were both significantly suppressed during the immediate recovery period following EHS (Fig 8 A & B). This is expected based on the lower plasma glucose seen during these time periods. Interestingly, both in the exercise sham controls and throughout recovery, females had consistently higher levels of plasma insulin and c-peptide compared to males. In contrast, we could measure no elevations in plasma glucagon in male mice at any time point. In females, glucagon was present during the later recovery period. *These data suggest some abnormalities in glucose metabolism, particularly in male mice, during the recovery from EHS. Even though glucose levels were lower during 0.5-3 hr recovery, one would normally expect a robust glucagon response when insulin levels are low. The data has the appearance of revealing a deficient pancreatic enzyme response immediately post EHS. We speculate that this may reflect the reduction in blood flow to the pancreas during EHS due to ischemia in the splanchnic circulation.*

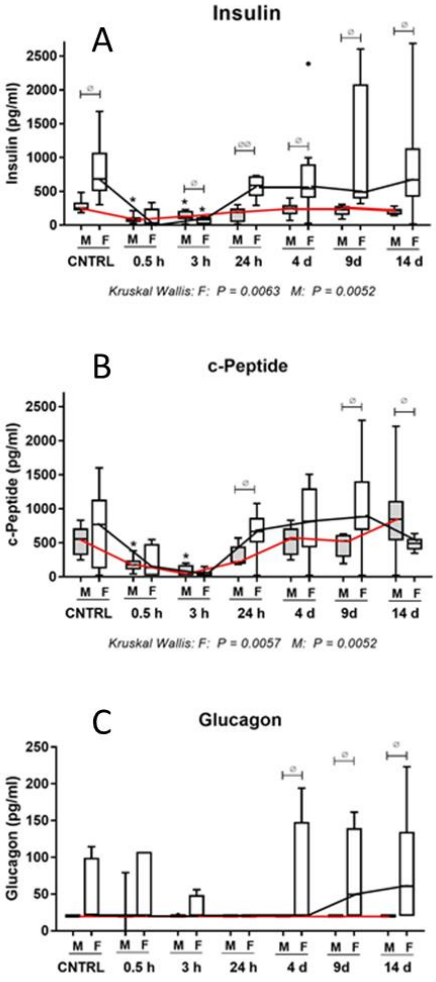


Figure 8. Plasma pancreatic enzyme responses during recovery from EHS. Red lines connect the medians in male, black lines are females. During 0.5-3 hr recovery all hormones were suppressed. Following EHS females demonstrated significantly higher values at most time points. θ = difference between male/female; * = difference from cntr.

Figure 9 illustrates the responses of two metabolic hormones, generally attributed to adipose tissue (adipokines), leptin and resistin. Previous studies have demonstrated that resistin levels are much higher in control females but leptin levels are higher in males (2). Our results are consistent with these findings in the control mice, resistin (Fig. 9A) and leptin (Fig 9B). The response patterns were also significantly different between males and females and demonstrated strikingly different patterns during EHS recovery. The best known function of resistin is that it reduces insulin sensitivity in tissues (9), though it also has recently been described as a secreted stress protein and chaperone, that may provide some protection to heat stress (8, 11). Resistin became acutely elevated at the 0.5 h recovery

period in males but showed little nor no change in females. From that point on resistin remained significantly lower in males than females. Leptin levels, were higher in males than females. This is not too unexpected since male C57/Bl6 mice have a higher percent fat content than females throughout life (3, 10). The females tend to rebound during the 1-4 days, post EHS, which corresponds to a period of weight gain in these mice (data not shown). The metabolic effects of leptin are largely associated with regulation of food intake, but recently it has been shown that leptin has the capacity to reduce thermal conductance (4).

We were also able to compare the metabolomics profiles between males and females (metabolomics data) at one time point following EHS, 3 hrs post vs. control. A more detailed metabolic profile follows below for the male population through the entire recovery period. In most measurements, the 3 h and control time points were not different in terms of the metabolic profiles between males and females in the plasma or in heart. However, there was one striking finding. The acylcarnitine abundance (an indicator of ongoing fatty acid metabolism in the heart) was reduced in females compare to male mice in the sham controls (Figure 10). In males,

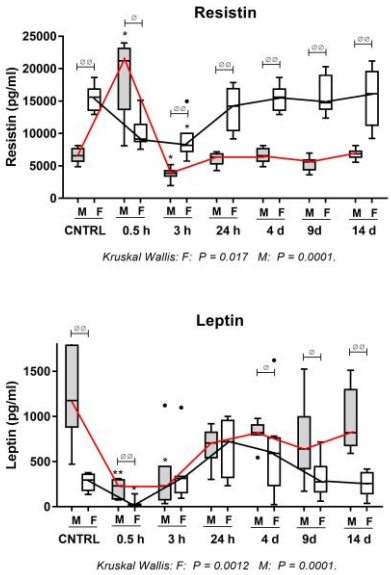


Figure 9. Adipose derived metabolic hormones, A) Resistin and B) Leptin during the recovery period from EHS.

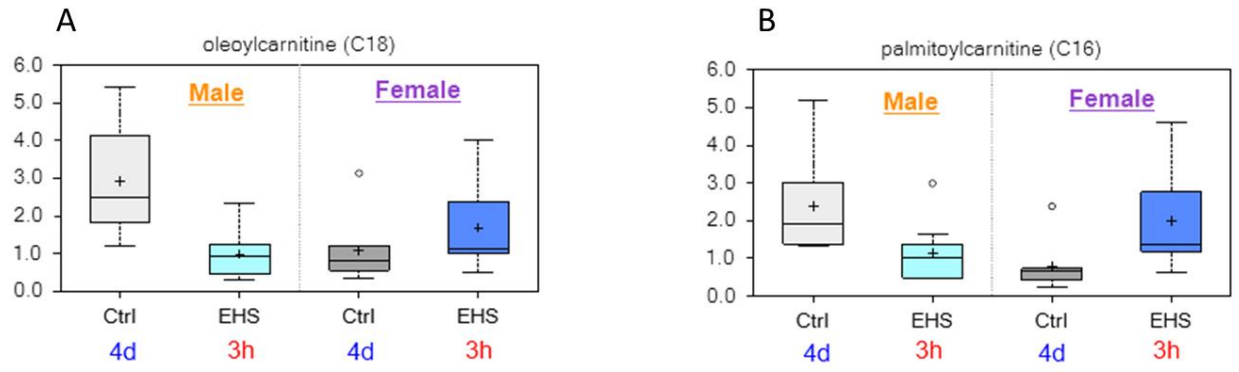


Figure 10. Changes in acylcarnitine abundance between male and female mice. (Metabolon data). Only the 4d control land 3h post EHS data were studied between male and female mice. See text for details.

these levels were higher at rest (control) but switched drastically lower after EHS suggesting a switch to mitochondrial fatty acid metabolism. In contrast in females, there was a low acylcarnitine abundance at rest but a modest trend toward an elevation at 3h post EHS. Since this was accompanied by what is considered control levels of acetylcarnitine supply, it suggests that females were actively metabolizing fatty acids in the heart at rest but males were metabolizing relatively less. It also suggests that following EHS, males switched and relied more heavily on fatty acids. These observations are confirmed in discussion below.

Differences in Cytokine Responses between male and female mice.

Plasma samples were evaluated for cytokine concentrations using an inflammation Luminex multiplex panel (27 cytokines). These analyses were performed at USARIEM. In general, the cytokines seen in the

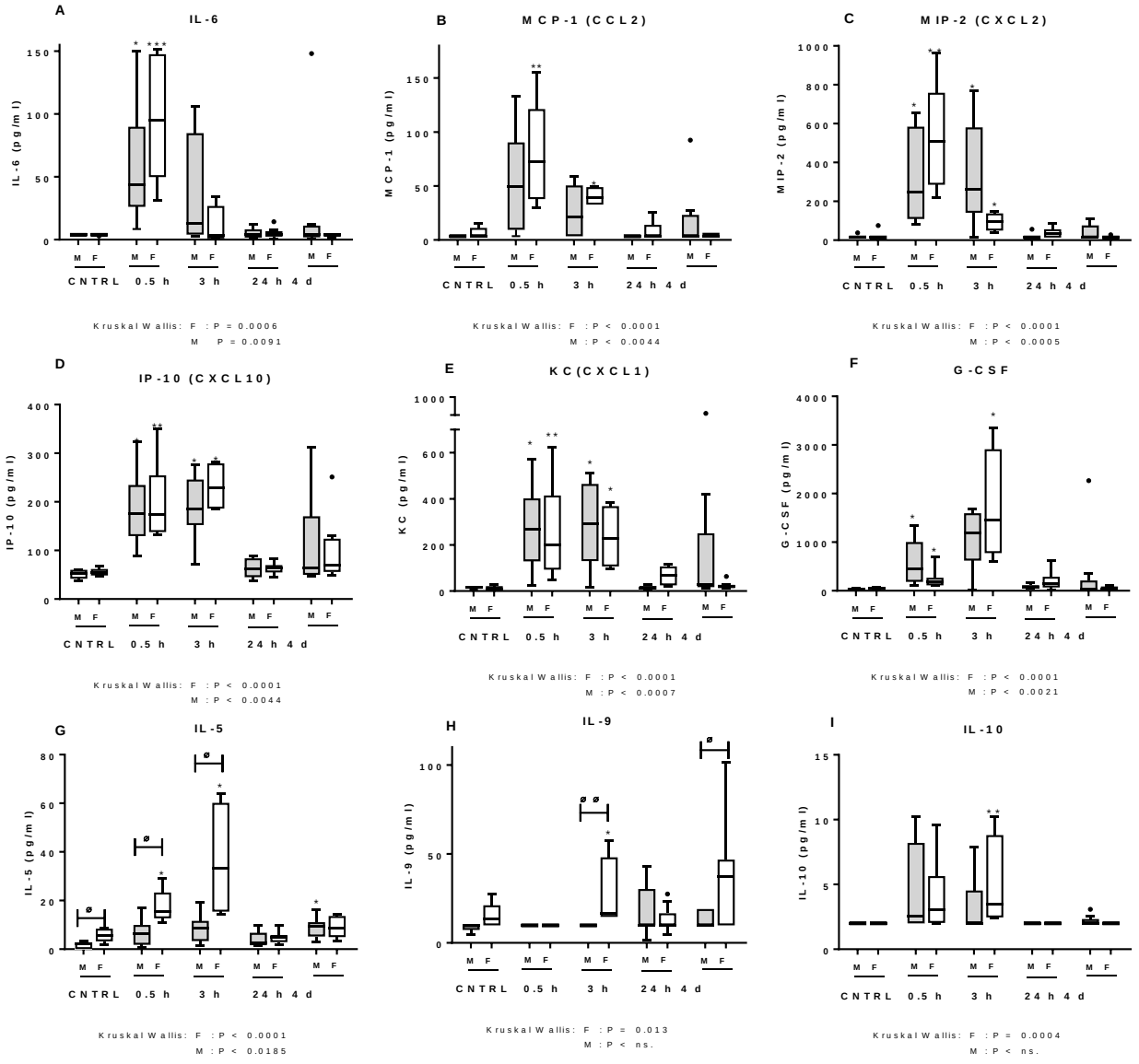


Fig. 10. Plasma cytokine measurements in male and female mice over the course of 0-4 d recovery from EHS. Most cytokines returned to normal or near normal by 4 days post. Data expressed as "whisker plots" because data was nonparametric. The lines represent the median values and the error bars are 95% Cis. Single dots are outliers (left in the analysis) * represent differences from sham 4 day control, θ = differences between males and females. (*in preparation for publication)*

plasma during the recovery period had similar patterns between males and females. Furthermore, the overall pattern in males was nearly identical to our previous publication in male mice (5). In general, in

females the cytokine and chemokine responses appeared more robust and tended to be sustained longer, but most of these apparent changes did not reach statistical significance. Interestingly, IL-5 and IL-9 were expressed in plasma and higher levels in females than males. We have never before observed elevations in these two cytokines in animal models of heat stroke. However, differences in the cytokine responses in heat may contribute to the observation that the incidence of acute allergic reactions to exertion in the heat, though relatively rare, occurs at a rate of 15 fold higher in female humans compared to males (12) . We are trying to understand the implications of this. *The data suggests that the inflammatory responses of male and female mice to EHS are similar and therefore it appears to exclude the hypothesis that immunosuppression (perhaps from elevated glucocorticoid secretion, is responsible for better heat tolerance in female compared to male mice.*

Metabolomic Responses in male mice during recovery from Heat stroke.

From plasma and ventricular muscle samples submitted to Metabolon for analysis through Danielle Ippolito and our colleagues at USACEHR with our collaborators at over 1500 chemicals were identified in both samples. The analyses is extremely complex and secondary analyses are ongoing. Based on summaries provided by Metabolon, the following chief conclusions are described Fig. 11. The early recovery period following EHS, was accompanied by significant changes in cellular energetics. The mice were initially hypoglycemic in the immediate post-EHS period, but appeared to compensate for this by

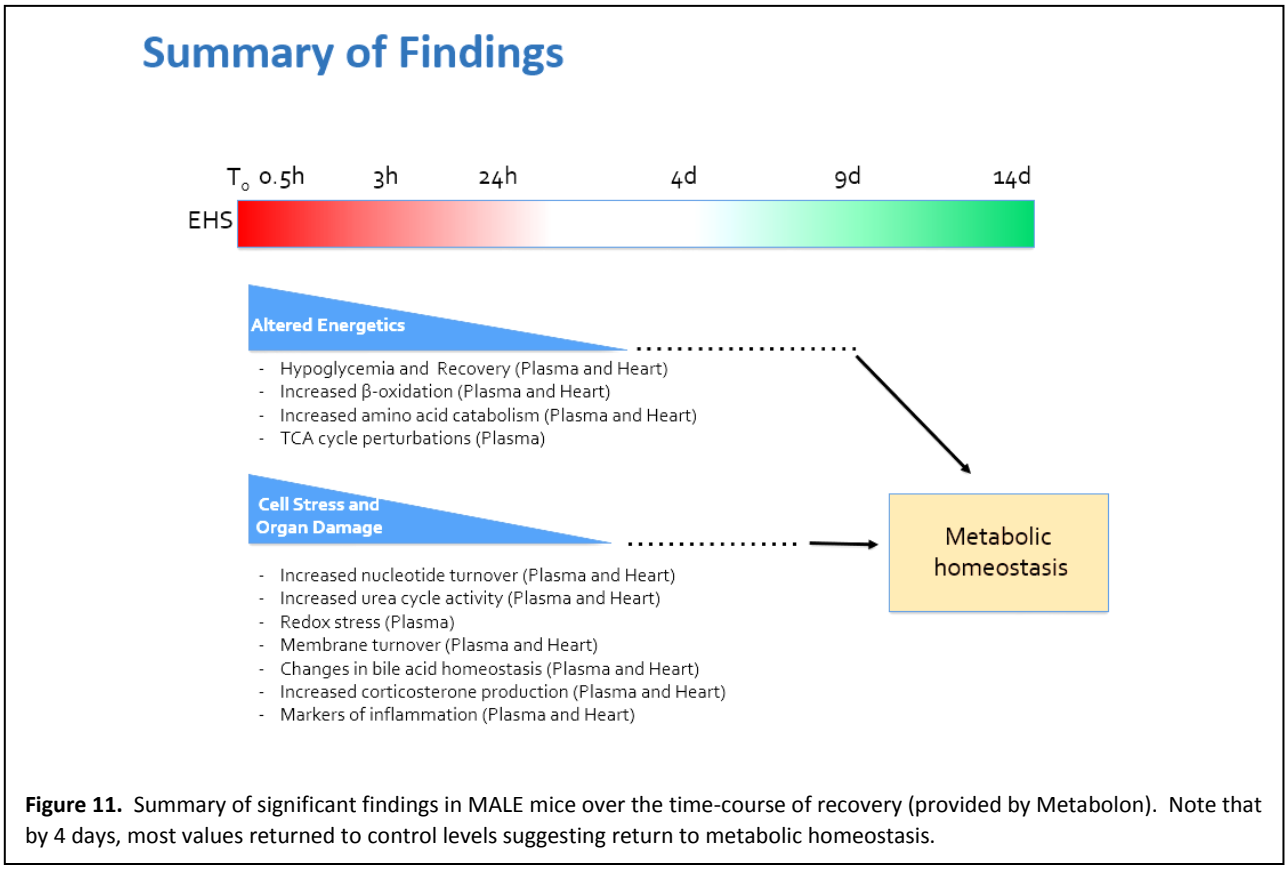
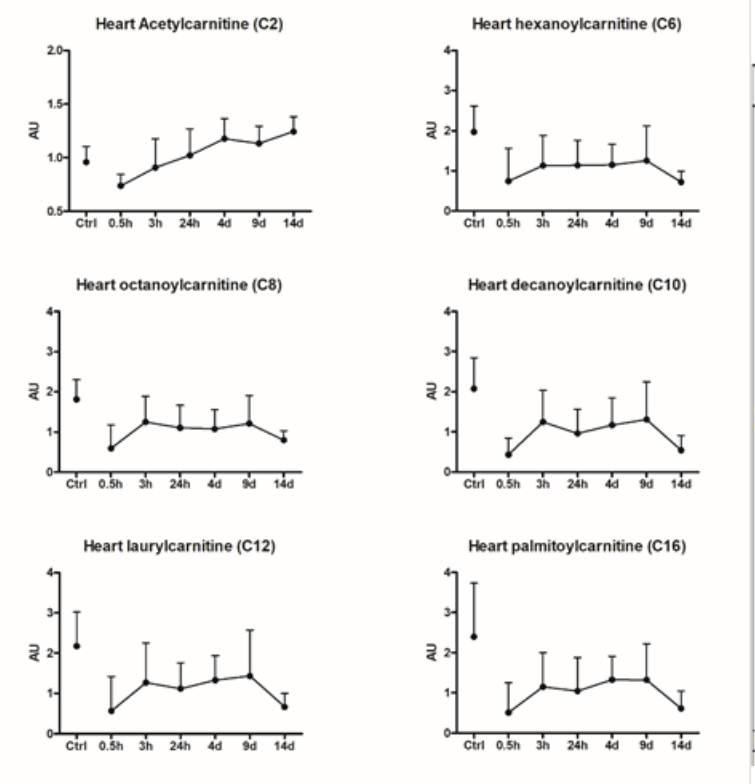


Figure 11. Summary of significant findings in MALE mice over the time-course of recovery (provided by Metabolon). Note that by 4 days, most values returned to control levels suggesting return to metabolic homeostasis.

increasing their rates of lipid oxidation and by utilizing amino acids as fuel sources. In parallel with these changes, the mice also exhibited changes in a number of other catabolic processes (*e.g.,* the urea cycle and nucleotide degradation pathways). Markers of oxidative stress were also altered as was the stress

hormone corticosterone (confirming results in fig. 6) and select inflammation-related metabolites. These latter responses, notably, are likely reflective of the cellular damage and/or organ dysfunction that accompanies EHS. Moreover, these responses appeared to be well conserved across the two genders. While some features persisted over several days, the majority of changes appeared to resolve at or before the 4 day recovery time point. Collectively, these findings provide a platform for further investigating how organisms respond to and recover from EHS."

One extremely interesting finding is illustrated in Figure 12. It is the only finding that separates EHS mice at the 14 day time-period from controls. In examining the heart of male mice throughout recovery and



		Welch's Two-Sam					
		Male Post-EHS Group (Time) Male Ex Control (4d)					
Sub Pathway	Biochemical Name	0.5h	3h	24h	4d	9d	14d
Fatty Acid Metabolism (Acyl Carnitine)	acetylcarnitine (C2)	0.77	0.95	1.06	1.23	1.18	1.3
	3-hydroxybutyrylcarnitine (1)	0.67	1.22	0.49	0.78	0.91	0.76
	3-hydroxybutyrylcarnitine (2)	0.38	0.66	0.47	0.66	0.73	0.43
	valerylcarnitine (C5)	0.38	0.5	0.2	0.38	0.5	0.62
	hexanoylcarnitine (C6)	0.38	0.57	0.58	0.58	0.64	0.37
	octanoylcarnitine (C8)	0.33	0.69	0.61	0.59	0.67	0.44
	decanoylcarnitine (C10)	0.21	0.6	0.45	0.56	0.63	0.26
	cis-4-decenoylcarnitine (C10:1)	0.45	0.52	0.62	0.44	0.43	0.07
	laurylcarnitine (C12)	0.26	0.58	0.51	0.61	0.66	0.31
	myristoylcarnitine (C14)	0.23	0.48	0.47	0.57	0.62	0.24
	palmitoylcarnitine (C16)	0.21	0.48	0.44	0.55	0.55	0.25
	palmitoleoylcarnitine (C16:1)*	0.15	0.34	0.39	0.5	0.54	0.22
	stearoylcarnitine (C18)	0.51	0.66	0.61	0.68	0.68	0.46
	linoleoylcarnitine (C18:2)*	0.18	0.36	0.54	0.54	0.5	0.25
	linolenoylcarnitine (C18:3)*	0.19	0.32	0.43	0.48	0.49	0.18
	oleoylcarnitine (C18:1)	0.13	0.33	0.44	0.55	0.53	0.26
	myristoleoylcarnitine (C14:1)*	0.2	0.43	0.51	0.59	0.6	0.26
	arachidoylcarnitine (C20)*	0.09	0.32	0.46	0.57	0.62	0.32
	arachidonoylcarnitine (C20:4)	0.71	0.86	0.94	0.96	0.91	0.8
	adrenoylcarnitine (C22:4)*	0.35	0.5	0.4	0.4	0.67	0.35
	behenoylcarnitine (C22)*	0.2	0.51	0.76	1.02	0.97	0.53
	dihomo-linolenoylcarnitine (20:3n3 or 6)*	0.22	0.49	0.38	0.46	0.54	0.32
	dihomo-linoleoylcarnitine (C20:2)*	0.04	0.12	0.09	0.16	0.18	0.1
	eicosenoylcarnitine (C20:1)*	0.04	0.23	0.26	0.32	0.36	0.21
	erucoylcarnitine (C22:1)*	0.06	0.28	0.3	0.37	0.38	0.26
	docosadienoylcarnitine (C22:2)*	0.04	0.04	0.04	0.05	0.04	0.07
	docosahexaenoylcarnitine (C22:6)*	0.99	0.8	1.26	0.81	0.92	0.75
	lignoceroylcarnitine (C24)*	0.42	0.57	1.15	1.21	1.01	0.97
	margaroylcarnitine*	0.35	0.6	0.66	0.62	0.69	0.38
	nervonoylcarnitine (C24:1)*	0.36	1.3	0.97	0.88	1.16	0.85
	pentadecanoylcarnitine (C15)*	0.26	0.48	0.56	0.58	0.68	0.28
Ketone Bodies	3-hydroxybutyrate (BHBA)	1.12	2.15	0.59	0.84	0.88	1.02
Free Fatty Acid	*Total FFA*	0.55	0.76	0.83	0.65	0.47	0.65

Figure. 11. Interesting metabolomics results showing a sustained elevation in β oxidation flux in the ventricles of mice that is sustaine for over 15 days. The graphs on the left are the relative means and SDs of some representative acycarnitine and acetylcarnitine. The Table on the right shows significance using non parametric statistics. The boxes in green color are significantly reduced; the boxes in red color are significantly elevated. *Stats provided by Metabolon. Manuscript in preparation.*

at 14 days, they persisted in having a pronounced and significant change in the way they handled fatty acid oxidation in the ventricle. What you see graphically on the left upper panel (heart acetylcarnitine) is gradually increasing and is significantly elevated from 4-14 days (red boxes on the right upper table). However, nearly every intermediate of β-oxidation (acylcarnitines) shown in the other 5 graphs is significantly reduced compared to controls. This means that the heart has undergone a shift in mitochondrial utilization of fatty acids such that this metabolic pathway is amplified and undergoing a large flux of carbon units. We feel this is the most significant finding of all of the metabolomics data we are working with. *It tells us that a single bout of EHS induces "switch" in energy metabolism towards more β-oxidation that lasts at least 14 days.* Is this maladaptive or adaptive? That is something we

hope to discover in future experiments. We also believe we can link epigenetic responses discussed below to these findings.

Results of Ibuprofen Effects on the GI tract during EHS.

We have completed the first round of the ibuprofen studies in both males and females, though all the data and histological specimens are still being analyzed. The hypothesis is that ibuprofen administration will make the mice more susceptible to heat stroke and they will have a greater amount of organ injury, particularly to the GI tract. Our data is analyzed for females at this time, however, males appear to have the same response pattern. Surprisingly, the female mice ran longer and acquired greater speeds when exposed to ibuprofen (Fig. 12). Therefore under ibuprofen, mice appeared to be more heat tolerant. However, in the Ibuprofen treated animals, there was a significant increase in both injury to the stomach and the intestine. As shown in Figures 13 and 14. Ibuprofen alone caused modest damage compared to heat + ibuprofen.

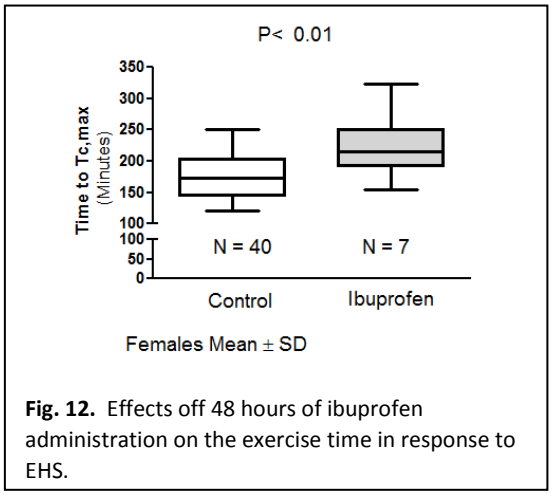
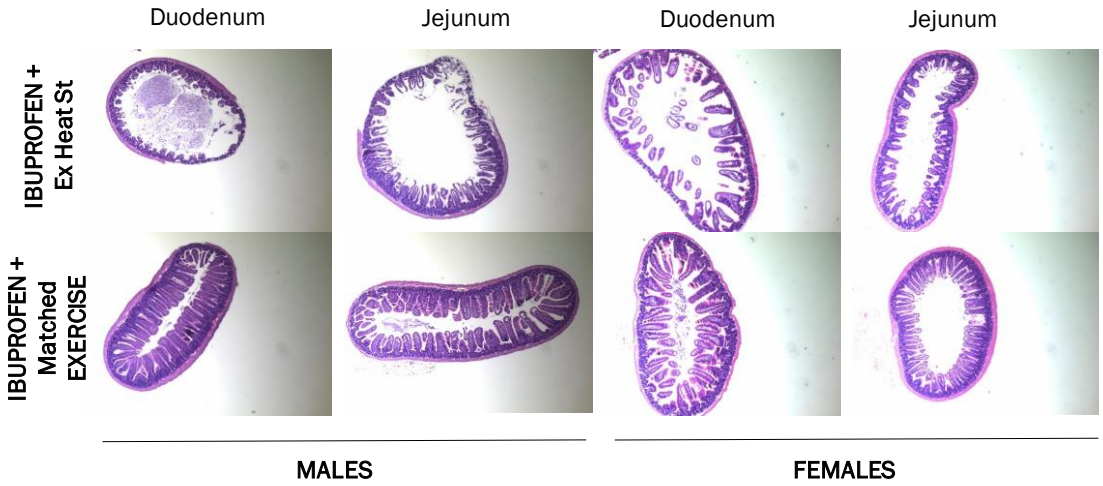


Fig. 12. Effects off 48 hours of ibuprofen administration on the exercise time in response to EHS.



Small Intestine

IBUPROFEN + EXERCISE CONTROLS: Moderate damage, shortened villi

IBUPROFEN + EXERTIONAL HEAT STROKE: Extreme villus injury, shortened or denuded villi

No damage to the colon in either treatment

Figure 13. Examples of small intestinal injury in animals exposed to ibuprofen at therapeutic levels (top row) and then exposed to EHS (3 hours post). These are compared to minor injury seen in control animals exposed to ibuprofen alone (bottom row). Results demonstrate massive denuding of intestinal villi throughout the small intestine. *Unpublished data.*

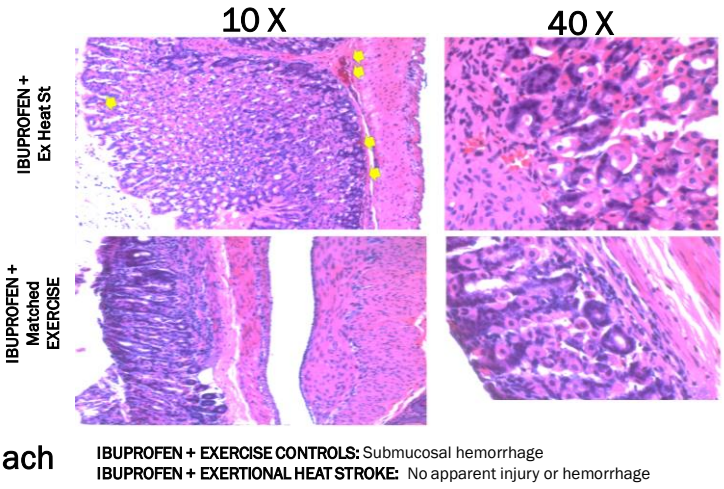


Figure 14. Stomach lesions induced by Ibuprofen administration prior to EHS and in control animals. Lesions were identified by the presence of blood outside of blood vessels.

Preliminary work on establishing reliable molecular biomarkers for maladaptive responses to EHS

We continue to seek molecular markers that help identify responses to EHS that will individuals more susceptible to subsequent heat stroke and/or lead to long term complications of EHS. We submitted nuclear DNA from 4 EHS animals, 4 days after heat stroke and compared against 4 sham controls at the same time point after matched exercise. Extremely impressive differences in DNA methylation patterns were identified in the EHS exposed animals. Hundreds of genes were both hyper- and hypo-methylated compared to controls (Fig. 15). The genes that were altered were involved with Ca^{+2} regulation, heat shock proteins, metabolic enzymes, and cytokine regulation. Secondary pathway analyses were performed by Rasha Hammamieh and colleagues at USACEHR and they have delivered those results to us. We are currently repeating these measurements at 30 days and coupling them to phenotype measurements consistent with heat stroke susceptibility. So far, 50% of the animals at 30 days have a disorder in Ca^{+2} regulation in skeletal muscle identified from isolated soleus muscles. This disorder resembles the kind of responsiveness seen in malignant hyperthermia. This was the basis of a recently submitted White Paper that we hope will lead to continued interest in this model and our work and its application to humans.

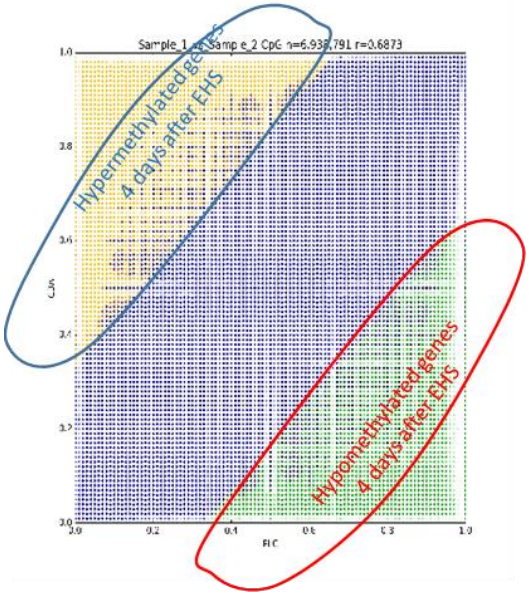


Fig. 15. Summary of statistically significant genes that were hypermethylated or hypomethylated , 4 days after EHS compared to matched exercise control mice at 4 days.

What opportunities for training and professional development did the project provide?

Because of this support, we were able to provide training opportunities for Alex Mattingly MS, who was supported for part of the year on the project. We were also able to use this support to employ two MS students in our Department, Christian Garcia and Gerard Robinson who have now converted to a full time Ph.D. program in spring of 2017. Both students are minority students. All three students have first author abstracts for the Experimental Biology Meeting in Chicago of 2017 and at least two will be attending ACSM in Minneapolis. Finally, we provided training for our postdoc, Dr. Orlando Laitano. He remains only been partially funded by this project but has not only provided the senior guidance in the lab but also developed a new line of research (funded by our endowment) which is looking at the molecular sources of rhabdomyolysis in heat, with and without coexisting hypertonic stress (relevant to heat stress and heat injury in the US Military). This is related to this study but not supported by this project. He is currently first authoring the metabolomics work. Through our collaborations with USARIEM, some junior scientists on the team have played critical roles, most notably Michelle King and Shauna Dineen, who will receive benefits from the interaction and the manuscripts that will emerge from this work.

How were the results disseminated to communities of interest?

- The P.I. presented the preliminary findings of these studies at Ft Detrick on October 19-20 in 2016 at the Extreme Environments Research in Progress Review.
- Two abstracts have been presentated at the 2017 Experimental Biology Meetings in Chicago.

  Gerard P. Robinson, Michelle A. King, Alex J. Mattingly, Christian K. Garcia, Orlando Laitano, David Van Steenbergen, Lisa R. Leon, Thomas L. Clanton "**Major Metabolic Hormone Responses to Exertional Heat Stroke in Mice." FASEB J.**

  Christian K. Garcia, Gerard P. Robinson, Alex J. Mattingly, Orlando Laitano, David Van Steenbergen, Michelle A. King, Lisa R. Leon, Thomas L. Clanton "**Differences in tolerance to exertional hyperthermia between male and female mice" FASEB J**

- In January a new manuscript was published based on the preliminary work for this project with collaboration between USAIREM and UF. King, MA, Leon, LR, Morse, DA, Clanton, TL. "**Unique cytokine and chemokine responses to exertional heat stroke in mice."** J Appl Physiol, 1:122(2) 296-306, 2017

- In August of 2017 two abstracts were presented at the MHSRS Symposium in Orlando, Fl.

  Thomas L. Clanton, Michelle Kin, and Lisa Leon. **The intestinal epithelium is vulnerable to heat, exercise and NSAIDs.**

  Orlando Laitano, Brian Ingram, Christian Garcia, Gerard Robinson, Alex Mattingly, Danielle Ippolito, Lisa Leon, Thomas L Clanton **Single exposure to exertional heat stroke results in a sustained metabolic switch to lipid oxidation in heart ventricular muscle of male mice.**

What do you plan to do during the next reporting period to accomplish the goals and objectives:

We are currently expanding the ibuprofen experiments with an additional cohort of animals to be certain that the effects of Ibuprofen + EHS are over and above EHS alone. We cannot detect that with our current groups. We continue to study the effects on histological markers of GI injury, which takes time and involves a number of lab members. As soon as we finish these groups we wills submit the plasma and heart samples for metabolomics and lipidomics measurements, in collaboration with USACEHR.

We are continuing to develop the preliminary data for molecular biomarkers, most notably the epigenetic biomarkers. We are only able to support pilot work for these experiments but we hope to complete sufficient data to make it a compelling direction for further research. This work continues to be collaborative between USCEHR and USARIEM. .

Within the next few months we will begin the naproxen and a H2S containing naproxen studies. The company that makes the naproxen derivative wants us to start with a diclofenac derivative first and that may delay the completion of all of the aims, but we think we can get this work done by the end of the funding period.

At the moment our biggest job is to complete the writing of the manuscripts from the first two years of work. We are well on our way on two new manuscripts with a good idea for how to package the third. We hope to have these submitted around Jan of 2018.

We are submitting abstracts on this work for presentation at Exp Biol in San Diego (spring, 2018) and different ones for ACSM. The title of this work is pending, but it will involve both the NSAID work, the metabolomics work and some new analyses of the male/female differences.

4. IMPACT

Impact on the Field. This model has become extremely refined and predictable and we believe it will stand the test of time as the first go-to model for preclinical studies in EHS research. We continue to be surprised by new findings that are not expected from other models such as passive heat stroke. The NSAID studies provide some insights we did not expect, particularly the ability of mice to withstand a greater exertional heat load. We feel this could impact the way the Armed Services approaches guidelines for NSAID usage. We also feel work done on the H2S-containing NSAID during EHS may encourage military medicine to proceed to utilization of some of these drugs which are now finishing Phase II clinical trials.

Impact on other Disciplines: We have confidence that biomarkers we can identify may be applicable across other fields, particularly with respect to studies underway on epigenetic markers. We also are of the opinion that our work identifies a unique “stress induced immune response” which can be separated from classic innate immunity. This may ultimately impact the field of immunology.

Impact on technology transfer: Nothing to report

Impact on Society beyond science and technology: It is possible that our work will impact the evaluation and treatment of exertional heat stroke patients. However, at this time, it is premature to

predict how this will be manifest itself. We have found the differences between male and female mice, from a metabolic and hormonal aspect to be remarkable. These striking differences may help to understand health related questions between the sexes that are not related to EHS but involve the same integrative physiological systems.

5. CHANGES OR PROBLEMS

We have had no significant problems performing this work and because of excellent help in the laboratory we are ahead of schedule in data collection. We have much work to do on analysis, as expected. We did run into a ACURO/IACUC issue in that the principle investigator was unaware he needed approval for each student that was added to the protocol. This was corrected and an investigation conceded it to be a misunderstanding but a violation. There have been no other major changes to the animal use protocols except for the mechanism for delivery of ibuprofen.

Our expenditures are in line with expectations and we should be able to complete the studies over the next year without interruption.

. **6. PRODUCTS:**

- abstracts submitted for presentation at the 2017 Experimental Biology Meetings in Chicago.

  Gerard P. Robinson, Michelle A. King, Alex J. Mattingly, Christian K. Garcia, Orlando Laitano, David Van Steenbergen, Lisa R. Leon, Thomas L. Clanton "**Major Metabolic Hormone Responses to Exertional Heat Stroke in Mice." FASEB J. 31(1) suppl 1085.a**

  Christian K. Garcia, Gerard P. Robinson, Alex J. Mattingly, Orlando Laitano, David Van Steenbergen, Michelle A. King, Lisa R. Leon, Thomas L. Clanton "**Differences in tolerance to exertional hyperthermia between male and female mice" FASEB J 31(1) suppl 1018.10**

- King, MA, Leon, LR, Morse, DA, Clanton, TL. **Unique cytokine and chemokine responses to exertional heat stroke in mice.** J Appl Physiol, 1:122(2) 296-306, 2017

7. PARTICIPANTS AND OTHER COLLABORATING ORGANIZATIONS

Individuals who have worked on the project.

Personnel at UF.

Name: Thomas Clanton Ph.D.
Project Role: P.I.
Researcher Identifier Orchid: 0000-0003-0600-7150
Nearest person-month worked 3.5 Person Months
Contribution to project : All aspects of the project.
Funding support: Univ of Florida

Name: Orlando Laitano, Ph.D.
Project Role: Postdoctoral fellow
Researcher Identifier
Nearest person-month worked: 2 Person Months
Contribution: Data collection, planning design of experiments, directing other lab personnel.
Funding support: Rest of support from the National Institutes of Health

Name: Alex Mattingly, MS
Project Role Senior Graduate Student/Research Assistant
Researcher Identifier
Nearest person-month worked 3 Person Months
Contribution: Oversees surgeries, data collection and managing activities and training of other personnel.
Funding support: Univ of Florida Research Assistantship.

Name: Christian Garcia
Project Role: Graduate student research assistant
Researcher Identifier
Nearest person-month worked 8 Person Months
Contribution: Ran most of the training and EHS experiments, collected specimens, animal care, histology
Funding support: Entirely from this award.

Name: Gerard Robinson
Project Role: Graduate student research assistant
Researcher Identifier
Nearest person-month worked: 6 Person Months
Contribution: Ran training and EHS experiments, collected specimens, animal care, histology
Funding support: Entirely from this award.

Name: Deborah Morse
Project Role Technician
Researcher Identifier
Nearest person-month worked: 2 Person Months
Contribution: Assisted in animal care, performed some biochemical experiments, laboratory management
Funding support: Supported in part by three other large NIH grants from the PI and two other investigators.

***Has there been a change in the active other support of the PI since the last reporting period*.**

None:

What other organizations were involved as partners:

Organization Name: USARIEM (Lisa Leon, primary contact, Michelle King)
Location of Organization: Natick MA.
Contribution to the Project:

Financial Support: USARIEM receives separate financial support for their part of the project. I have never received a report of the amounts distributed for this purpose.
The role of USARIEM is to evaluate samples for metabolic hormones and cytokine expression. The also collaborate and plan experiments, help with writing manuscripts and data analysis.

Organization Name: USACEHR (Danielle Ippolito, primary contact, currently left the program. Our current contact is Valerie T. Divito)
Location of the Organization: Frederick MD
Contribution to the Projects:

Financial Support: USARIEM receives separate financial support for their part of the project. I have never received a report of the amounts distributed for this purpose.
UASCEHR helps evaluate samples for metabolomic, lipidomic and proteomic markers. They help with bioinformatics and interpretation of results and writing of manuscripts.

APPENDIX MATERIAL

1. References
2. Quad Chart for 4th Quarter 2016-2017
3. Publication King et al. 2017
4. Abstracts submitted Garcia et al., Robinson et al. published in Federation Proceedings, 2017.

APPENDIX 1: References:

1. **Armed Forces Health Surveillance B**. Update: Heat injuries, active component, U.S. Army, Navy, Air Force, and Marine Corps, 2015. *MSMR* 23: 16-19, 2016.
2. **Gui Y, Silha JV, and Murphy LJ**. Sexual dimorphism and regulation of resistin, adiponectin, and leptin expression in the mouse. *Obes Res* 12: 1481-1491, 2004.
3. **Hong J, Stubbins RE, Smith RR, Harvey AE, and Nunez NP**. Differential susceptibility to obesity between male, female and ovariectomized female mice. *Nutr J* 8: 11, 2009.
4. **Kaiyala KJ, Ogimoto K, Nelson JT, Muta K, and Morton GJ**. Physiological role for leptin in the control of thermal conductance. *Mol Metab* 5: 892-902, 2016.
5. **King MA, Leon LR, Morse DA, and Clanton TL**. Unique cytokine and chemokine responses to exertional heat stroke in mice. *J Appl Physiol (1985)* 122: 296-306, 2017.
6. **King MA, Leon LR, Mustico DL, Haines JM, and Clanton TL**. Biomarkers of multi-organ injury in a pre-clinical model of exertional heat stroke. *Journal of Applied Physiology accepted with minor revision*: 2015.
7. **Leon LR, DuBose DA, and Mason CW**. Heat stress induces a biphasic thermoregulatory response in mice. *Am J Physiol Regul Integr Comp Physiol* 288: R197-R204, 2005.
8. **Lu DY, Chen JH, Tan TW, Huang CY, Yeh WL, and Hsu HC**. Resistin protects against 6-hydroxydopamine-induced cell death in dopaminergic-like MES23.5 cells. *J Cell Physiol* 228: 563-571, 2013.
9. **Muse ED, Obici S, Bhanot S, Monia BP, McKay RA, Rajala MW, Scherer PE, and Rossetti L**. Role of resistin in diet-induced hepatic insulin resistance. *J Clin Invest* 114: 232-239, 2004.
10. **Reed DR, Bachmanov AA, and Tordoff MG**. Forty mouse strain survey of body composition. *Physiol Behav* 91: 593-600, 2007.

11. **Suragani M, Aadinarayana VD, Pinjari AB, Tanneeru K, Guruprasad L, Banerjee S, Pandey S, Chaudhuri TK, and Ehtesham NZ**. Human resistin, a proinflammatory cytokine, shows chaperone-like activity. *Proc Natl Acad Sci U S A* 110: 20467-20472, 2013.
12. **Vadas P, Sinilaite A, and Chaim M**. Cholinergic Urticaria with Anaphylaxis: An Underrecognized Clinical Entity. *J Allergy Clin Immunol Pract* 4: 284-291, 2016.

Prevention of Organ Injury in Exertional Heat Stroke: Preclinical evaluation of a new class of NSAIDs

Log Number: #14267001 FY 16

W81XWH-15-2-0038 BAA Extramural Medical Research

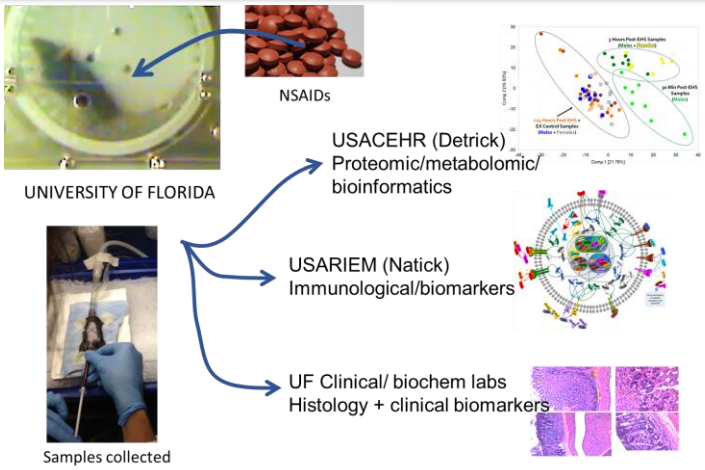
PI: Thomas L Clanton **Org: University of Florida** **Partners: USARIEM, USACEHR** **Total Award Amount: $265 (3rd yr)**

Study/Product Aim(s)

- to define the time course of multi-organ injury, repair and recovery of metabolic control in exertional heat stroke (EHS)
- to determine sex differences in susceptibility to EHS in mice
- to identify metabolomic and proteomic biomarkers that define underlying disorder in EHS
- to test the impact of commonly used NSAIDs on susceptibility to organ injury in EHS
- to test the effectiveness of new H_2S-containing NSAIDs on reducing intestine and organ damage in EHS

Approach

Instrumented and exercise-trained mice (♂ &♀) run on a running wheel within an incubator (37.5°C) until symptom limited (neurological). Samples of blood and various organ systems are taken at intervals up to 14 days and prepared for proteomic, metabolomic and genomic analysis. In upcoming experiments, the animals will be given different varieties of NSAID to determine susceptibility to organ injury.



Accomplishments: Completed EHS studies on 112 (♂ &♀) mice. Completed the metabolomics and lipidomics analyses in collaboration with USACEHR and USARIEM. Completed physiological analyses of the response to heat in ♂ &♀ . Completed first series of ♂ &♀ mice exposed to ibuprofen and EHS.

Timeline and Cost

Activities / CY	15	16	17	18
Collection of tissues from EHS studies in male and female mice				
Proteomic/metabolomic/and immunological analysis of samples				
Test effects of common NSAIDs on organ injury in EHS				
Effects of new generations of H2S containing NSAIDs in EHS				
Estimated Budget ($K)		**$325K**	**$265K**	**$268K**

Note: October start date in '15 so budget listed in fy 17'

Updated: 010/30/2017

Goals/Milestones

CY15 Goal – ☑ purchase equipment, train personnel begin EHS

CY16 Goals – ☑ Complete male EHS and control experiments with 6 time points of EHS recovery ☑ Submit male samples to USACEHR and USARIEM ☑ Complete female mice EHS studies

CY17 Goal –☑ submit remaining EHS samples to USACEHR and USARIEM ☑ Begin studies of effects of predominant NSAIDs on organ injury in EHS. ☑ Begin studies of NSAID-H_2S drug studies ☑ Completed metabolomics and lipidomics analyses. ☐ Writing manuscripts on male-female difference and metabolomics responses to EHS.

CY18 Goal –Complete NSAID-H_2S studies and analyze and write up data

Comments/Challenges/Issues/Concerns

- Experimental model working well, there are no major problems.
- Have completed data collection for entire first and 2nd year, part of yr 3

Budget Expenditure to Date

Projected Expenditure: $401,799 (by end of year 3)

Actual Expenditure: $89,403 (07/15/2017) $185,000 direct costs remain FY3

J Appl Physiol 122: 296–306, 2017.
First published December 1, 2016; doi:10.1152/japplphysiol.00667.2016.

RESEARCH ARTICLE

Unique cytokine and chemokine responses to exertional heat stroke in mice

Michelle A. King,[1] Lisa R. Leon,[2] Deborah A. Morse,[1] and Thomas L. Clanton[1]

[1]*Department of Applied Physiology and Kinesiology, College of Health and Human Performance, The University of Florida; and* [2]*Thermal and Mountain Medicine Division, United States Army Research Institute of Environmental Medicine, Natick, Massachusetts*

Submitted 25 July 2016; accepted in final form 28 November 2016

King MA, Leon LR, Morse DA, Clanton TL. Unique cytokine and chemokine responses to exertional heat stroke in mice. *J Appl Physiol* 122: 296–306, 2017. First published December 1, 2016; doi:10.1152/japplphysiol.00667.2016.—In heat stroke, cytokines are believed to play important roles in multiorgan dysfunction and recovery of damaged tissue. The time course of the cytokine response is well defined in passive heat stroke (PHS), but little is known about exertional heat stroke (EHS). In this study we used a recently developed mouse EHS model to measure the responses of circulating cytokines/chemokines and cytokine gene expression in muscle. A very rapid increase in circulating IL-6 was observed at maximum core temperature ($T_{c,max}$) that peaked at 0.5 h of recovery and disappeared by 3 h. IL-10 was not elevated at any time. This contrasts with PHS where both IL-6 and IL-10 peak at 3 h of recovery. Keratinocyte chemoattractant (KC), granulocyte-colony-stimulating factor (G-CSF), macrophage inflammatory protein (MIP)-2, MIP-1β, and monocyte chemoattractive factor-1 also demonstrated near peak responses at 0.5 h. Only G-CSF and KC remained elevated at 3 h. Muscle mRNA for innate immune cytokines (IL-6, IL-10, IL-1β, but not TNF-α) were greatly increased in diaphragm and soleus compared with similar measurements in PHS. We hypothesized that these altered cytokine responses in EHS may be due to a lower $T_{c,max}$ achieved in EHS or a lower overall heat load. However, when these variables were controlled for, they could not account for the differences between EHS and PHS. We conclude that moderate exercise, superimposed on heat exposure, alters the pattern of circulating cytokine and chemokine production and muscle cytokine expression in EHS. This response may comprise an endocrine reflex to exercise in heat that initiates survival pathways and early onset tissue repair mechanisms.

NEW & NOTEWORTHY Immune modulators called cytokines are released following extreme hyperthermia leading to heat stroke. It is not known whether exercise in hyperthermia, leading to EHS, influences this response. Using a mouse model of EHS, we discovered a rapid accumulation of interleukin-6 and other cytokines involved in immune cell trafficking. This response may comprise a protective mechanism for early induction of cell survival and tissue repair pathways needed for recovery from thermal injury.

interleukin-6; CXCL1; granulocyte-colony-stimulating factor; exercise; hyperthermia

EXERTIONAL HEAT STROKE (EHS) is a life-threatening condition where the body is no longer able to dissipate the heat load produced during physical exertion. This can lead to extreme elevations in core temperature (T_c), central nervous system dysfunction, and subsequent multiorgan damage (7). This condition affects seemingly healthy individuals, such as military personnel, occupational workers, and athletes, making this illness even more enigmatic. While EHS is distinct from passive heat stroke (PHS) (35), the etiologies of both conditions are still poorly understood, and although multiorgan dysfunction is common in both (35, 38, 39, 53), the extent to which they share underlying mechanisms is not known. Despite efforts to prevent multiorgan damage via rapid cooling, many individuals still succumb to multiorgan failure. Furthermore, for those individuals who survive the initial heat injury, 40% are more likely to die earlier in life than their matched counterparts (62). To develop clinical interventions and prevent long-term organ damage, it is important to understand the underlying causes responsible for multiorgan injury.

The multiorgan dysfunction that occurs as a consequence of heat stress has been suggested to be the result of excessive inflammatory processes, where cytokines serve as important mediators (38, 56). The local response to tissue damage involves the production of cytokines at the injury site, which, with the help of chemokines, function in attracting lymphocytes, neutrophils, and monocytes to aide in the healing process (69). PHS models, as well as hyperthermia itself, display an acute rise in cytokines with dominant elevations in interleukin-6 (IL-6), interleukin-10 (IL-10), and interleukin-1β (IL-1β) and a lesser rise in tumor necrosis factor-α (TNF-α) (12, 30, 39). Importantly, the circulating cytokine pattern following PHS is unique from that seen following exposure to endotoxin or acute exercise (39, 49, 64, 67). However, the circulating cytokine pattern following EHS has yet to be determined.

One of the distinct differences between PHS and EHS is the role of the exercising muscle. Exercising muscle is not only the main contributor to increases in T_c during physical activity but also has the ability to act as an endocrine organ, contributing cytokines, particularly IL-6, to the circulation (49, 58). Furthermore, skeletal muscle has been shown to be responsive to heat stress following PHS (64). However, the role of the skeletal muscle in contributing to the circulating cytokine profile is not known in EHS.

To understand the acute cytokine responses to EHS, our objective was to determine the pattern of cytokines and chemokines expressed in the circulation and the expression of select cytokines in skeletal muscle throughout the course of EHS and recovery. Because there may be a cumulative effect of hyperthermia, exercise, and other potential factors such as endotoxemia or release of catecholamines, we hypothesized that the stress-induced cytokine response to EHS would be greater in magnitude but follow a similar time course as that observed in

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Downloaded from http://jap.physiology.org/ by 10.220.33.5 on March 1, 2017

PHS. We predicted that the additional stress of exercise would exacerbate the associated cytokine and chemokine profile.

METHODS

Animal care. All animal protocols were approved by the University of Florida Institutional Animal Care and Use Committee. In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals" as prepared by the Committee for the Update of the *Guide for the Care and Use of Laboratory Animals* of the Institute for Laboratory Animal Research, National Research Council. Ninety-five mice were used for data collection in this study. A subset of these mice had been used previously to determine multiorgan dysfunction in EHS (35). All were C57BL/6J males (Jackson Laboratories, Bar Harbor, ME) weighing an average of 29.1 ± 3 (SD) g, approximately 4 mo of age. Mice were housed in groups until they were implanted with telemetry devices, after which they were individually housed in 7.25 in. wide × 11.75 in. deep × 5 in. high cages lined with Harlan corn cobb bedding and maintained on a 12:12-h light-dark cycle at 20–22°C/30–60% relative humidity (RH). A standard chow diet (LM-485m Envigo; Teklad, Madison, WI) and water were provided ad libitum until the EHS protocol. Experiments were performed in the morning of the light cycle (~0700–1000).

Animal preparation and training. As described previously (35), under isoflurane anesthesia, mice were implanted with temperature telemetry transmitters (TA-E-Mitter; Starr Life Sciences, Oakmont, PA) for monitoring T_c. The mice were allowed to recover with subcutaneous buprenorphine injections every 12 h for 48 h and then recovered undisturbed for >2 wk. Following this recovery period, exercise wheels and enrichment huts (Silent Spinner and Small Animal Igloo Hideaways; PETCO, San Diego, CA) were introduced in the cages for 3 wk. During this period, mice had ad libitum access to the running wheel throughout the day and night. On the 3rd wk, additional exercise training/acclimation was implemented to familiarize the mice to the environmental chamber in the laboratory (Thermo-Forma 3940 Incubator; Thermo-Fisher, Waltham, MA) and to the customized forced running wheel system (model 80840; Lafayette Instrument, Lafayette, IN). The first exercise session in the chamber consisted of 15 min of freewheeling, where the mouse was free to run and explore their surroundings. This was followed by a short recovery period (<5 min). Next, mice were started at an initial speed of 2.5 m/min and then increased by 0.3 m/min every 10 min for 60 min. Training sessions on the next two consecutive days consisted of only the incremental protocol for 60 min. At the fourth and final session, the same protocol was used, but exercise time and incremental speed were elevated until the animals exhibited fatigue. Fatigue was defined as refusal to run or walk on the wheel for >5 s. No shock or any other manual stimuli were used to maintain running speed.

EHS. Following the last training session, mice were given 2 days of rest with free access to the running wheel in their cages. The evening before or the morning of the EHS test, mice were brought to the laboratory in their own cage. T_c was monitored with a data acquisition system, averaged over 30-s intervals (VitalView; Starr Life Sciences). After at least 2 h of resting data in the environmental chamber, each mouse was monitored until T_c dropped to <37.5°C for >15 min. At this time, the environmental temperature (T_{env}) and RH were increased to 37.5°C, 50% RH; water, food, and the cage lid were removed leaving only the wire rack exposed. This T_{env} was based on previous work where we studied EHS at three different T_{env} (between 37.5 and 39.5) and RH values (35–90%) (35). At this temperature, the animals' exertional heat production had the greatest contribution to overall heat load and therefore had the greatest potential for distinguishing differences from PHS. As soon as the environmental chamber equilibrated to the target T_{env} (~1 h), the chamber was opened, and the animal was quickly placed in the running wheel. The forced running wheel protocol was then initiated. The mouse's behavior was monitored continuously in real time with a video camera. Running speed began at 2.5 m/min and increased 0.3 m/min every 10 min until the mouse reached a T_c of 41°C, which served as threshold beyond which the running speed was kept constant (Fig. 1, *A* and *B*). The end point of the EHS test was "symptom limited," since nearly all mice (≈98%) displayed a sudden loss of consciousness and collapse. However, reaching a T_c of 42.5°C was also considered a humane end point but was a rare occurrence. At the end of the protocol, T_{env} was adjusted back to room temperature, the chamber door was opened, and the mouse was carefully watched until it regained consciousness. At this time, it was weighed and returned to its home cage. T_c continued to be monitored for a 24-h recovery or until death at an earlier time point (described below). The 12-h light-dark cycle was maintained in the environment during the recovery period.

EHS experiments. Five groups of mice were studied (n = 6–9/group) to determine the time course of cytokine expression. Mice were euthanized at 80 min into the protocol (which was set to be ≈0.5 h before $T_{c,max}$) at $T_{c,max}$ and 0.5, 3, and 24 h post-$T_{c,max}$. At each time point, blood and tissue samples were collected. Five other groups of sham controls (EXC) were treated identically without heat exposure, and tissues were sampled at the same times. These mice were exercised at the average time and intensity of the EHS mice (maximum speed: 5.2 m/min, duration: 113 min) but with the environmental chamber maintained at 25°C and 50% RH (35).

For sample collection, the mice were placed under isoflurane anesthesia, and blood samples were obtained by transthoracic cardiac stick. Soleus, gastrocnemius, and diaphragm were removed for later biochemical or histological analyses. Thoracotomy and heart removal were performed under deep anesthesia.

Tissue and blood samples were obtained from another group of naïve control mice (NC) that did not undergo surgery, any exercise training, any specific enrichment, or any exercise or heat interventions (n = 6).

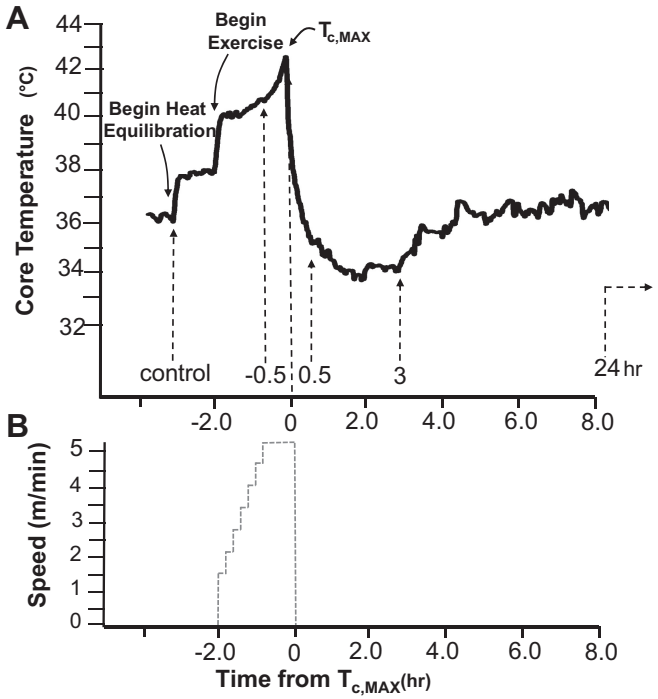

Fig. 1. *A*: typical core temperature (T_c) profile for the exertional heat stroke (EHS) protocol, showing the intervals of blood/tissue collections relative to peak core temp ($T_{c,max}$). *B*: average forced running wheel time course, starting at 2.5 m/min, with 0.3 m/min until 40.5°C and then held at steady-state exercise until $T_{c,max}$.

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PHS experiments. Two more groups of animals were exposed to a PHS protocol. One set ($n = 6$) was exposed to 39.5°C at 30% RH, identical to previous approaches described by Leon and colleagues (40) except that the end point for these PHS mice was T_c of 42.1°C, rather than 42.4–42.7°, which was used for previous studies (40, 64). This end point temperature was used because it was the average $T_{c,max}$ acquired by the EHS mice in this study. This was done to determine if differences in response of EHS could be attributed to the lower peak T_c reached. We only took samples at the 3-h time point in these mice because this corresponds to a time when there is marked cytokine expression in PHS but a time when there is almost no circulating cytokine expression in EHS.

Another set of mice [matched PHS (PHS_m)] ($n = 6$) underwent a passive heating protocol designed to mimic the shortened thermal area (heat load) experienced in EHS groups. Thermal area was calculated as defined by Leon et al. (40), adapted from Hubbard et al. (32). Mathematically this equals approximately the area under the curve of the temperature profile for all points at which T_c was >39.5°C (units = °C·min). To obtain a very similar thermal area in PHS_m, the environmental temperature was elevated to 43.5°C/50% RH, determined by trial and error in a group of test mice. These mice were also studied at the single time point of 3 h post-$T_{c,max}$ for the same reasons identified in PHS mice.

Plasma cytokine measurements. Blood was collected, using heparin as the anticoagulant, and spun at 2,000 relative centrifugal force, and plasma (250 μl) was pulled off the buffy coat, separated into aliquots, and stored at −80°C. Plasma cytokines and chemokines were determined using a Luminex system, employing MILLIPLEX MAP Mouse cytokine/chemokine-premixed 25 plex assay kits, which include the antibodies for the following analytes: granulocyte-colony-stimulating factor (G-CSF), granulocyte macrophage-colony-stimulating factor, IFN-γ, IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-15, IL-17, interferon-γ-induced protein-10, keratinocyte chemoattractant (KC), monocyte chemoattractive factor-1 (MCP-1), macrophage inflammatory protein (MIP)-1α, MIP-1β, MIP-2, regulated on activation, normal T cell expressed and secreted (RANTES), and TNF-α. The test was performed according to the manufacturer's protocols, as described elsewhere (67).

RNA isolation, reverse transcription, and real-time PCR. To determine innate immune cytokine expression in skeletal muscle, the soleus, diaphragm, and gastrocnemius muscles were dissected and flash-frozen at the −0.5 h, $T_{c,max}$, 0.5-, 3-, and 24-h time points. As previously described (67) RNA was separated from DNA by bromochloropropane and precipitation in isopropanol. After a 75% ethanol wash and resuspension in DEPC water, purity of RNA was quantified by spectrophotometry. Total mRNA was reverse transcribed using a Verso cDNA Synthesis Kit. Preformulated Taqman Gene Expression assays were used for IL-1β, IL-6, IL-10, and TNF-α. Relative quantitative real-time reverse transcription-polymerase chain reaction was done using TaqMan Gene Expression Master Mix on a StepOnePlus. Hypoxanthine phosphoribosyltransferase was used as a housekeeping gene based on previous studies in which we observed the gene to be stable in hyperthermic myofibers and tissues (67). Changes in target gene expression were independent of changes in the level of mRNA for hypoxanthine phosphoribosyltransferase. Relative quantitation was calculated using the $\triangle\triangle C_T$ method as described previously (31).

Statistical analyses. Statistical analyses were performed using SAS JMP (Cary, NC) and Graphpad Prism (La Jolla, CA). The large majority of cytokine and mRNA data was nonparametric, and, therefore, Kruskal-Wallis was used for all ANOVAs. Post hoc tests were done with Dunn's multiple-comparison test for nonparametric comparisons. Central tendency and variance of data were expressed as medians ± 25–75% quartiles because of the nonparametric nature of the datasets. To determine the probability of type 1 error due to multiple comparisons, the Benjamini-Hochberg procedure for estimating false discovery rate was applied (6), using a cutoff of 0.15 as an acceptable false discovery rate.

RESULTS

Plasma cytokine and chemokine responses to EHS. We sampled plasma cytokines and chemokines at time intervals denoted on a typical EHS T_c profile in Fig. 1*A*. Cytokines such as IL-1β, IL-6, IL-10, and TNF-α, which are classically involved in the innate immunity, are elevated following heat stroke (10, 11, 39, 64). However, in this model of EHS, only IL-6 was significantly elevated at any time point over the course of EHS, reaching a peak at +0.5 h into recovery (Fig. 2*A*). This response was suppressed by 3 h and remained undetectable at 24 h. Sham exercise controls displayed no significant changes in IL-6 nor any of the cytokines measured in this study, at any time (Fig. 2*B*).

As shown in Fig. 3*A*, plasma chemokines, MCP-1, MIP-1β, and MIP-2 followed a similar trajectory seen for IL-6, where peak concentrations occurred at 0.5 h of recovery, disappearing

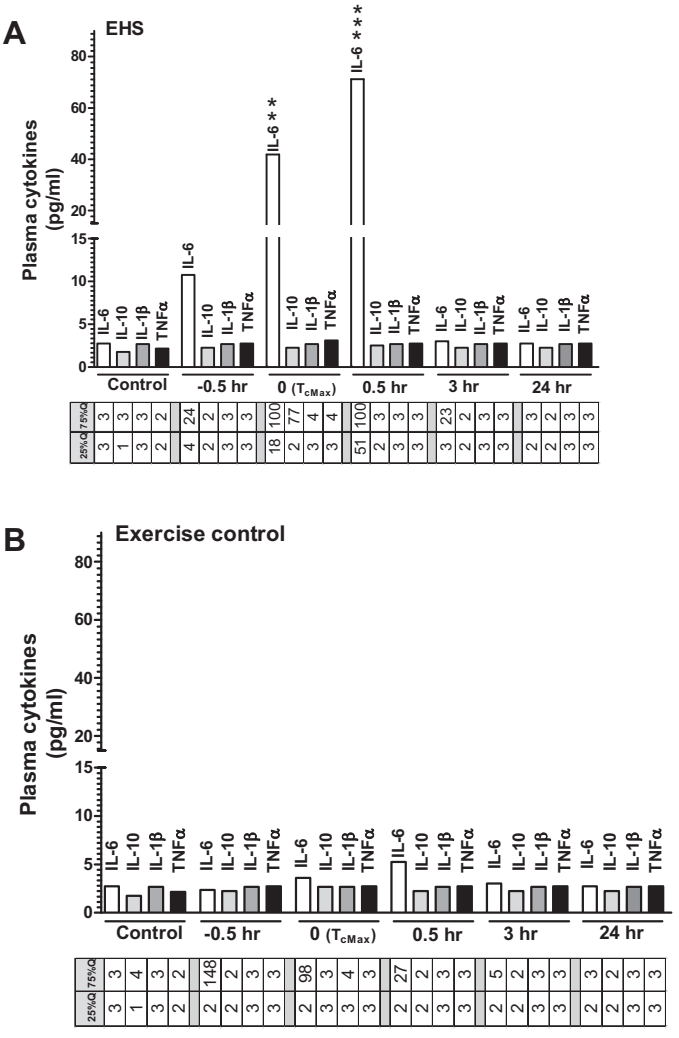

Fig. 2. Effects of EHS on common cytokines of innate immunity. *A*: responses of common innate immune cytokines to EHS. *B*: cytokine responses to sham exercise controls. Significance from naïve control: $P < 0.01$ (**) and 0.001 (**) (post hoc tests). MCP-1, monocyte chemoattractive factor-1; MIP, macrophage inflammatory protein; G-CSF, granulocyte-colony-stimulating factor; KC, keratinocyte chemoattractant. Benjamini-Hochberg procedure for multiple ANOVAs = false discovery rate (FDR) <15%. Bars = medians; tables below = 25–75% quartiles.

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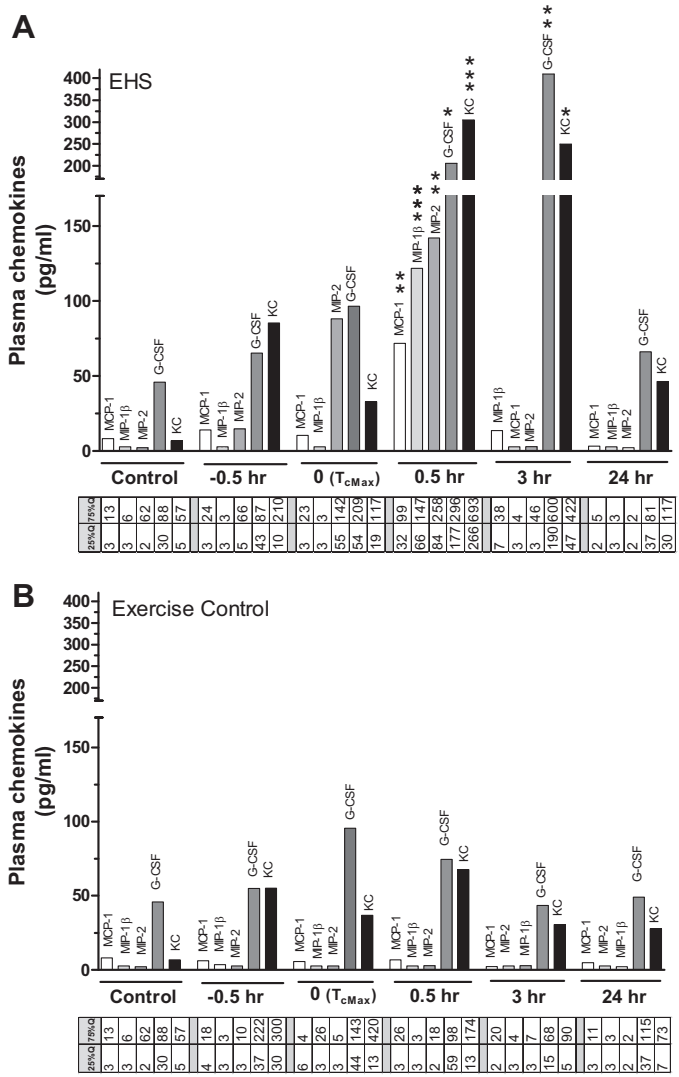

Fig. 3. Effects of EHS on chemokines and related cytokines. *A*: responses during and following EHS. *B*: responses to sham exercise controls. Post hoc significance from naïve control: $P < 0.05$ (*), 0.01 (**), and 0.001 (***) (post hoc tests). Benjamini-Hochberg procedure for multiple ANOVAs = FDR <10%. Bars = medians; tables below = 25–75% quartiles.

by 3 h (Fig. 2*A*). G-CSF and KC were also significantly elevated at 0.5 h but showed sustained or increasing levels at 3 h. G-CSF is not structurally classified as a chemokine but works synergistically with many other chemokines like KC to mobilize immune cells (68). All chemokines returned to control values by 24 h. There were no significant elevations in these chemokines in sham exercise controls (Fig. 3*B*). All other cytokines and chemokines tested with the multiplex array showed no significant elevation during EHS (data not shown). Refer to Table 1 for functional and structural classifications of responsive chemokines observed in this study.

PHS experiments. Previous PHS studies have shown that circulating IL-6 and IL-10 reach a peak response at 3 h of recovery (39, 64), with little or no response at $T_{c,max}$ and only modest responses at ≈0.5 h of recovery (64). To understand the origins of this delay in the PHS cytokine profile compared with the EHS profile, we tested several possible experimental mechanisms related to heat exposure.

First, because our EHS animals achieved an average symptom-limited $T_{c,max}$ of only 42.1°C [−0.3 to −0.6°C lower than the $T_{c,max}$ in studies by Leon and colleagues (39) and Welc et al. (64)], we repeated the standard PHS experiment in mice but stopped exposure when T_c reached 42.1°C. A typical temperature profile for this group (PHS) compared with EHS is shown in Fig. 4. Second, the PHS protocol resulted in an increased thermal area compared with EHS, averaging 409 ± 71°C·min in this series compared with 146 ± 30 (SD) °C·min in EHS. Therefore, we hypothesized that the altered cytokine response to EHS might reflect differences in the overall thermal load between PHS and EHS. To test this, we studied a second group of PHS animals (PHS_m) in which the thermal area was matched, using an elevated T_{env} in the chamber (43.5°C). This resulted in an average thermal area = 148 ± 20 (SD) °C·min (not significant from EHS). A typical thermal profile for PHS_m experiments is also shown in Fig. 4. We tested only the 3-h time point in these experiments because it represented a time when EHS cytokine responses were nearly absent in EHS but reached peak concentrations in PHS.

Comparisons of cytokines and chemokines between sham EXC, EHS, PHS, and PHS_m animals at the 3-h recovery point are shown in Fig. 5. In Fig. 5, *A–C*, are cytokine/chemokine responses to PHS that showed no response in EHS or EXC but were significantly elevated in PHS and PHS_m (i.e., IL-6, MIP-2, and RANTES). In Fig. 5, *D–F*, are cytokines/chemokines for which there were no responses in EHS, EXC, or PHS_m, but there were significant elevations in PHS. Both G-CSF and KC (data not shown) were significantly elevated in PHS and/or PHS_m and were not significantly different from EHS (data not shown). Elevations during EHS in these two chemokines are shown in Fig. 3.

Skeletal muscle innate immune cytokine gene expression. Skeletal muscle mRNA expression of IL-6, IL-10, IL-1β, and TNF-α was evaluated over the course of the EHS and EXC protocol through 24 h of recovery. The primary rationale was that significant muscle injury is associated with EHS but not PHS, based on plasma creatine kinase measurements (35) and unpublished observations of hindlimb motor dysfunction during recovery. In addition, in a previous study, the same approach was used in PHS at the similar time points, making comparison possible (64). Therefore, measuring the mRNA expression of important inflammatory cytokines in muscle can provide an indication of the timing of ongoing damage and repair processes in the muscle.

The results are summarized in Fig. 6 using samples from the whole gastrocnemius, soleus, and diaphragm. Results are expressed as fold change compared with samples taken from "naïve controls" that did not undergo surgery or acute exercise and were not exercise trained or exposed to heat. Note the tendency in early time points (−0.5 h to $T_{c,max}$) for cytokine mRNA to be suppressed before reaching $T_{c,max}$ (discussed below). There was very little mRNA response at any time point in gastrocnemius; however, in soleus and diaphragm, elevations in cytokine gene expression (IL-6, IL-1β, and IL-10) peaked at 0.5 h after $T_{c,max}$. IL-6 mRNA was also evident in diaphragm at $T_{c,max}$. These elevations in mRNA are 3–10 times higher than seen in comparable conditions and times during PHS (64). Note that TNF-α mRNA was not significantly elevated at any time point. Furthermore, in exercise controls, exercised to match EHS, and trained identically, there were no

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Table 1. *Functional-structural classes of chemokines/related cytokines observed in heat stroke*

Common Abbreviations	Name	Structure Name	Human Homolog	Observed in	Primary Functions
MCP-1	Monocyte chemoattractive factor-1	CCL2	Human MCP-1	EHS/PHS	Induces migration of monocytes and other immune cells
MIP-1β	Macrophage inflammatory protein-1β	CCL4	Human MIP-1β	EHS/PHS	Induces migration of monocytes and other immune cells
RANTES	Regulated on activation, normal T cell expressed and secreted	CCL5	Human RANTES	PHS	Stimulates T cells, basophils, and eosinophils
IP-10	Interferon-γ induced protein-10	CXCL10	Human IP-10	PHS	Induces migration of neutrophils, macrophages, and other immune cells.
MIP-2	Macrophage inflammatory protein-2	CXCL2	Human MIP-2 (90% IL-8 homolog)	EHS/PHS	Induces migration of neutrophils, macrophages, and other immune cells.
KC	Keratinocyte chemoattractant	CXCL1	IL-8 (similar to MIP2)	EHS/PHS	Stimulates hematopoietic and other stem cells and migration, similar to MIP-2
G-CSF	Granulocyte-colony-stimulating factor	CXC synergist	Human G-CSF	EHS/PHS	Not a chemokine but synergistic with CXCL1 and CXCL2; stimulating hematopoietic and stem cell release

PHS, passive heat stroke; EHS, exertional heat stroke.

significant elevations in muscle cytokine gene expression at any time point.

Based on the plasma cytokine results, we hypothesized that moderate acute exercise or the exercise training protocol itself may be responsible for suppression of cytokines. To test this, we compared our EXC group (which received enrichment and training sessions as previously described) with mice that were exposed to a single bout of moderate exercise, matched in timing and intensity to the EHS experiments. This experimental bout was preceded by only a familiarization trial the day prior, identical to the 60-min incremental training session that EXC mice received. We then measured inflammatory cytokine gene expression at 0.5 h of recovery because this time point displayed the greatest cytokine response in plasma. As shown in Fig. 7*A*, exercise suppressed IL-6, IL-1β, and IL-10 mRNA in the gastrocnemius and soleus but not in the diaphragm. Comparable trends were seen in the EXC (i.e., trained) animals, but fewer time points were statistically significant (Fig. 7*B*). The data are consistent with acute moderate exercise inducing an acute inhibition of inflammatory cytokine gene expression in skeletal muscle.

DISCUSSION

We have demonstrated that EHS results in cytokine/chemokine responses in plasma and skeletal muscle that are uniquely different in the timing, magnitude, and/or species compared with passive models of heat stroke. Contrary to our original hypothesis where we proposed the combined effects of exercise and hyperthermia would amplify the IL-6-induced response, circulating IL-6 emerges rapidly, reaching a peak level at 0.5 h of recovery and disappearing by 3 h, a point in time when the magnitude of circulating IL-6 is highest in PHS. Similar responses were seen for MIP-1β, MCP-1, and MIP-2, whereas G-CSF and KC increased rapidly but remained elevated at 3 h of recovery. At that time point, they were not different in magnitude from PHS. There was no evidence for elevations in circulating IL-10 at any time during recovery from EHS, whereas this is routinely elevated during recovery from PHS (Refs. 39 and 64 and Fig. 4).

Exploration of possible environmental variables related to the timing and magnitude of heat exposure failed to provide a suitable explanation for these phenomena. Therefore, the data suggest that the predominant experimental factor driving the rapid and unique cytokine/chemokine responsiveness of EHS is related to the influence of moderate forced exercise performed during hyperthermia. Neither matched exercise alone nor matched heat exposure alone could reproduce this pattern.

Possible origins of the cytokine/chemokine response pattern in EHS. There are several underlying stimuli that are thought to interact to produce the pattern of cytokine production seen in heat stroke that may be differentially affected by exercise in heat. One frequently mentioned stimulus is endotoxin or other pathogen-associated molecular patterns (PAMPs) released in the circulation from a leaky intestinal barrier (29, 56). How-

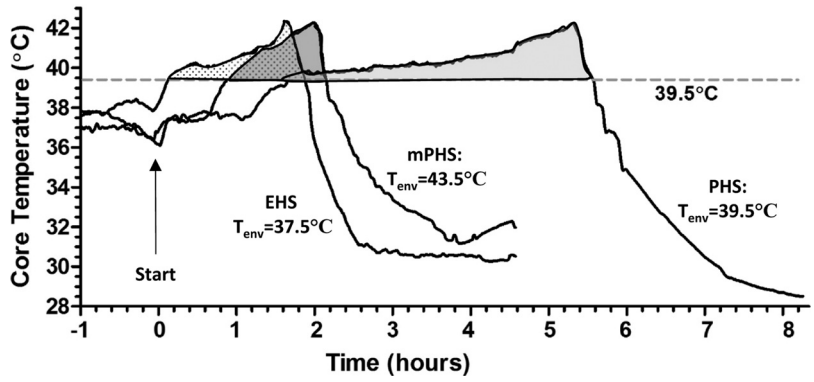

Fig. 4. Typical T_c profiles for EHS, passive heat stroke (PHS), and PHS at thermal area matched to EHS (PHS_m). Shaded areas represent the thermal areas (time·temperature >39.5°C). T_{env}, environmental temperature.

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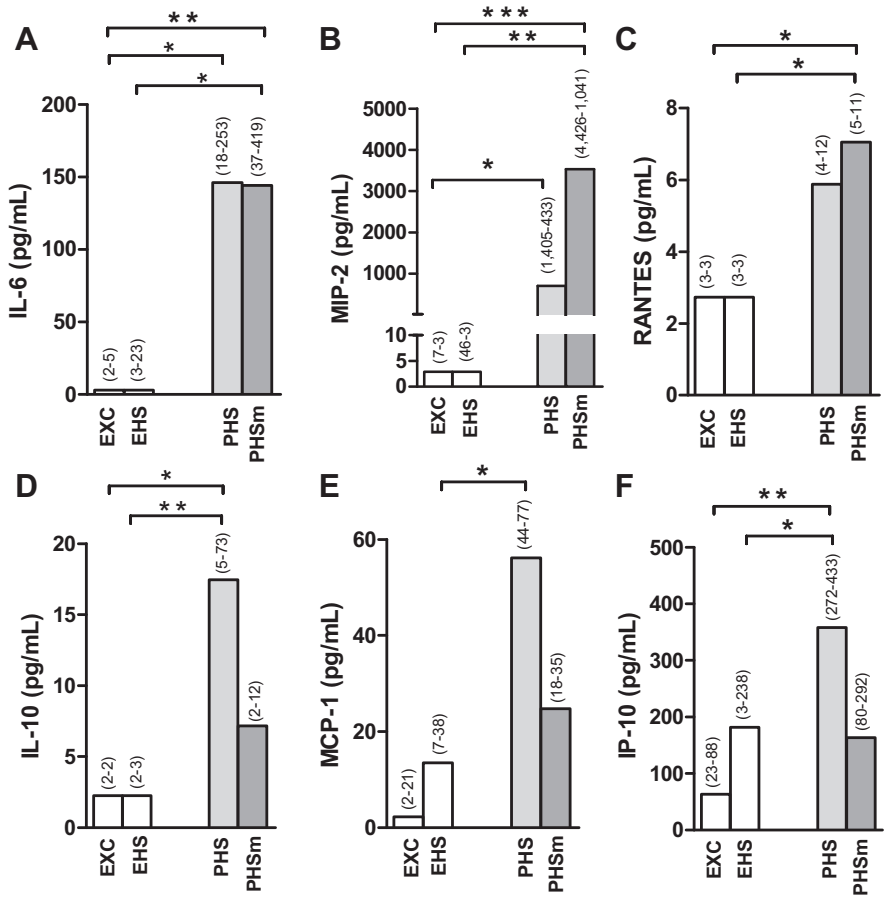

Fig. 5. Comparison of cytokines and chemokines significantly different at 3 h between EHS and models of PHS. EXC, sham controls. $P < 0.05$ (*), 0.01 (**), and 0.001 (***), Kruskall Wallis, Dunn's post hoc comparisons. Bars = median with 25–75% quartiles. Benjamini-Hochberg procedure for multiple ANOVAs = FDR <15%.

ever, the pattern of cytokines seen in the plasma during EHS is not typical of known cytokine responses to PAMPs, e.g., there is an absence of circulating TNF-α, IL-1β, or IL-12, at any time point. It appears more likely that the response is driven by a "stress-induced cytokine response" in which IL-6 is a predominant element. We have previously described this concept in the context of PHS in mice (64) where we observed altered expression of cytokine genes and Toll-like receptor isoforms that are uniquely different from the responses seen in the classic innate immune response. Its theoretical origins are based on observations of the response of isolated skeletal muscles to a variety of forms of cellular and systemic stress mediators (63, 65, 66).

Other possible influences that may contribute to the uniqueness of the PHS response include effects of intense endurance exercise alone, which produce rapid elevations in IL-6 and a variety of other cytokines and chemokines (47, 48). However, in paired exercise controls, there were no significant elevations in cytokines or chemokines. This may have been due to the moderate intensity of exercise. It is possible that hyperthermia amplified the exercise-induced IL-6 (52) as it does with other stimuli (66), but the exercise alone cannot account for the response.

Muscle injury is another potential factor. Local cytokines and chemokines produced following injury play important roles in tissue regeneration and repair (24, 59). Muscle injury was likely present in this model since elevations in plasma creatine kinase are present in this model of EHS but not PHS (35). In addition, Fig. 5 suggests ongoing inflammatory gene expression in both limb and diaphragm muscle during the recovery period that exceed by many fold what is seen in PHS (64). The responses appear to be local because mRNA for cytokines such as IL-1β and IL-10 are greatly upregulated in muscle, but these do not appear elevated in blood during the course of recovery. In addition, previous reports of the timing and magnitude of the circulating cytokine responses in the blood following muscle injury appear to be too small and slow to account for observations seen in EHS (59, 61).

Because the EHS animals received training sessions and had access to running wheels before EHS, this may have modified the cytokine responses during heat stroke. Previous studies have shown that endurance exercise training alters or dampens immune responsiveness (25, 45). It takes only 2 wk of voluntary wheel running in C57BL/6J mice to induce significant increases in heart-to-body mass ratio and percentage of oxidative fibers (1), suggesting that endurance training was likely in the mice provided running wheels. Resolving this variable will require a different approach, since mice unaccustomed to wheel running have more difficulty completing the EHS protocol and likely would experience much higher levels of psychological stress.

One important difference in the cytokine profile in EHS compared with PHS was the absence of circulating IL-10, at any time point. This was unexpected, since increases in circulating IL-10 are one of the most predictive circulating cytokines seen in human patients in heat stroke (9) and in animal

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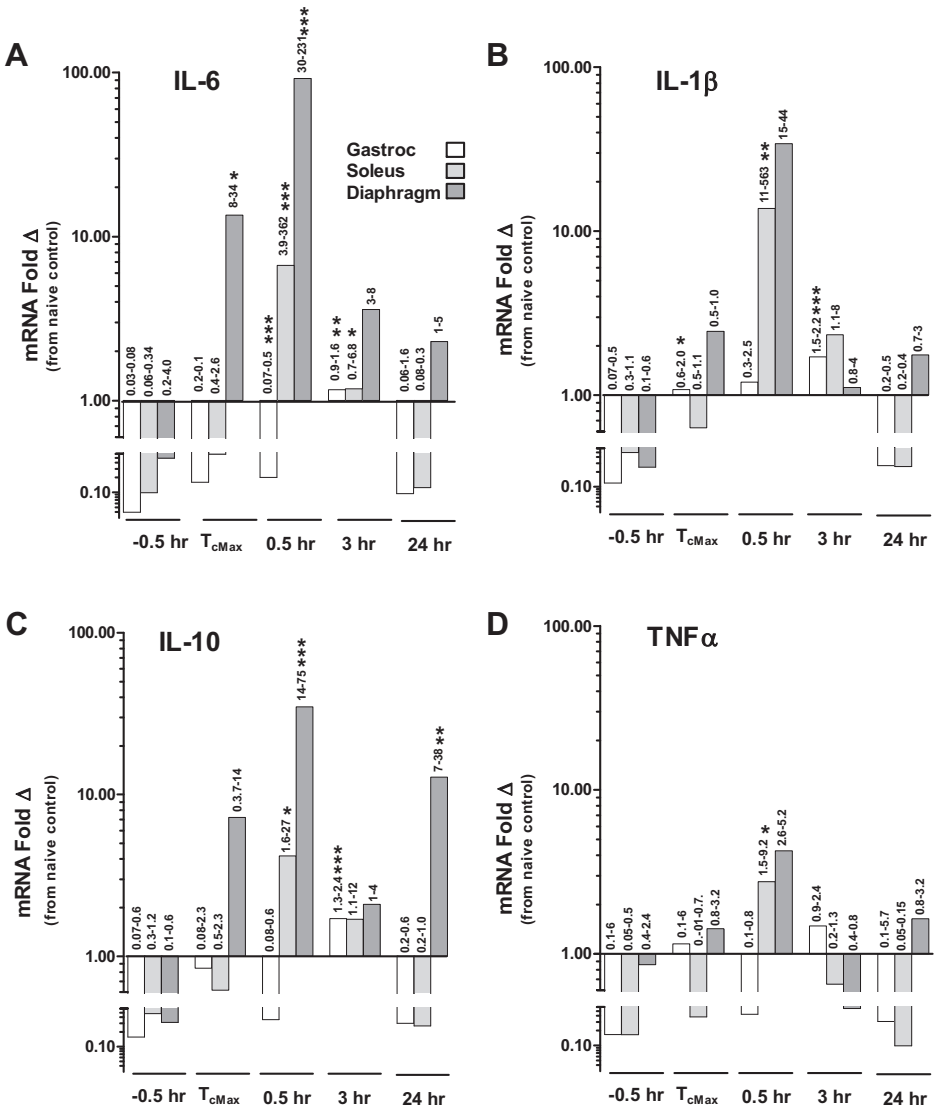

Fig. 6. Fold changes in innate immune cytokine mRNA in EHS gastrocnemius (gastroc), soleus, and diaphragm muscle. All changes reported relative to naïve control mouse muscle. Kruskal-Wallis ANOVA, Dunns post hoc: $P < 0.05$ (*), 0.01 (**), and 0.001 (***). Medians ± 25–75% quartiles. Benjamini-Hochberg procedure for multiple ANOVAs = FDR <15%.

models in PHS (10, 39, 64). Furthermore, IL-6 has been shown to be an important stimulus for IL-10 production (57), and intense exercise alone stimulates IL-10 (46). One possible explanation may reflect the effects of "forced" exercise on immune modulators such as corticosterone. In mice, during forced swimming exercise, corticosterone levels exceed 800 ng/ml within 5 min, approximately one-half of the value seen in parallel experiments in mice exposed only to passive heat (42°C) (26). In the mouse model for PHS, corticosterone has been shown to exceed 400 ng/ml, but this value is reached after ≈3 h of recovery (39). Although we did not measure plasma glucocorticoids in this setting, it is possible that forced running resulted in an early stress-induced surge in glucocorticoids that may have suppressed global cytokine gene expression. This could also explain the apparent suppression of muscle cytokine mRNA seen immediately after forced running (Fig. 6, *A* and *B*). Almost all cytokines and chemokines are suppressed by glucocorticoids, including IL-10 (19). Interestingly, one cytokine not affected appreciably by glucocorticoids is G-CSF (13), which turned out to be one of the most profoundly expressed plasma cytokines in EHS, rising rapidly in the circulation but continuing to rise up to 3 h.

A second important and unexpected finding was the very rapid emergence of IL-6, which was elevated in the plasma, at or shortly before $T_{c,max}$ (Fig. 1). This would seem to be too fast to reflect de novo protein synthesis, particularly when there appears to be simultaneous suppression of IL-6 mRNA (at least in muscle, Fig. 6). Most of the circulating chemokines also emerged during this time frame (Fig. 2). One possible mechanism is that these cytokines/chemokines were prestored in microvesicles or endosomes and were then released early in EHS. In mouse limb muscle, IL-6 is stored in such microvesicles and then released within 25 min from the beginning of an exercise protocol (37). Microvesicle or exosome release has also been shown in some systems to be facilitated by heat stress or by costimulation with other cytokines like IL-1β (18, 72). For example, in tumors, heat stress is a powerful stimulus for release of exosomes that contain many of the same CCL- and CXC-chemokine species we describe here (18). In theory, triggered release of prestored cytokines in this manner could supersede opposing immunosuppressive influences of glucocorticoids produced in the stress of exercise in the heat. This could be a kind of fail-safe acute endocrine stress response

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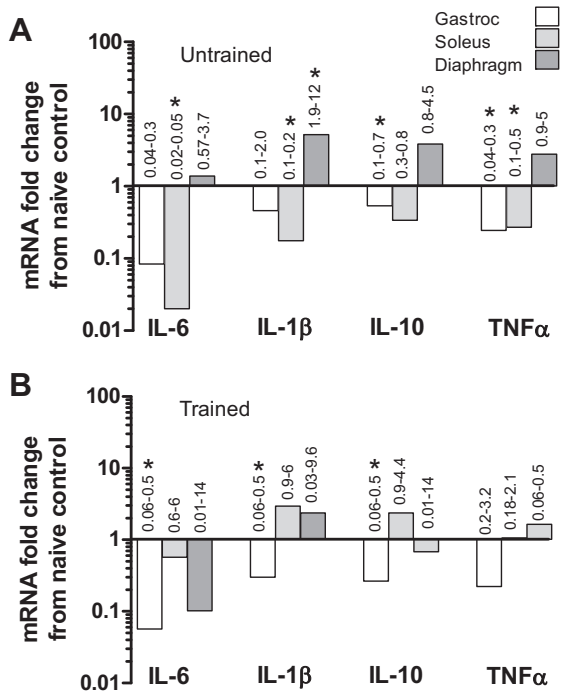

Fig. 7. Effects of a single bout of exercise (matched to EHS) on innate immune cytokine gene expression in muscle. Samples collected at 0.5 h post-$T_{c,max}$. *A*: untrained mice without cage running wheels or exercise training. *B*: response of EXC mice. Medians ± 25–75% quartiles. FDR = 0.15 using Benjamini-Hochberg procedure. *$P < 0.05$.

from tissues that could be important in recovery from acute illness.

Because of the large role muscle plays in exercise, we have focused on it as a source of circulating cytokines in EHS. However, it is highly plausible that other organs make significant contributions to the cytokine profile seen in EHS. Tissue damage resulting from heat stress may impart damage to the liver, kidney, heart, spleen, lung, small intestine, and brain as well as the skeletal muscle (8, 10, 23, 27, 35, 43). When these organs are damaged, they may release cytokines, or resident macrophages, dendritic cells, endothelial cells, or astrocytes may participate in the inflammatory response to injury. Therefore, although we did not directly measure other organs as potential sources of circulating cytokines, it is likely that they contribute to the cytokine profile seen in plasma.

Functional significance of the pattern of cytokine/chemokine production in EHS. In this model all experimental animals survived up to two weeks or to the point of sample collection. After a few hours of recovery, they show a remarkable ability to return to near-normal behavior, despite evidence of underlying organ damage (35). One of the primary functions of both cytokines and chemokines, besides defending against pathogens, is to participate in the process of wound healing and damage repair (69). This occurs, in part, through recruitment of peripheral blood mononuclear cells (PBMCs) and other immune cells in damaged tissue (59) but also by stimulation, recruitment, and mobilization of stem cell or progenitor cell populations in the bone marrow or other tissues (5, 42, 50).

In a previous study (51) we demonstrated that, in PHS, early injection of low levels of recombinant IL-6 enabled anesthetized mice to withstand hyperthermic temperatures for longer periods of time, to have protection from intestinal injury, and to demonstrate suppression of proinflammatory cytokines in the circulation. The protective influence of IL-6 in similar acute life-threatening conditions, or the loss of protection in knockout studies, has now been well established in a number of models, including hemorrhagic shock (2), sepsis (4, 41), acute pancreatitis (21), ischemic heart injury (22), and liver failure (20). Several mechanisms have been proposed but include pre- or postconditioning through Janus kinase/signal transducer and activator of transcription 3 signaling, promoting cell survival (22, 44, 55), upregulation of manganese superoxide dismutase in critical organs such as liver (14), activation of acute-phase response in liver (15), and stimulation of anti-inflammatory cytokines and cytokine receptors (60). We hypothesize that the early secretion of IL-6 and possibly chemokines in this model of EHS may have played an overall protective role in supporting survival and protection from multiorgan injury.

The specific sets of chemokines secreted may also have contributed to recovery from heat injury. There are two broad categories, as shown in Fig. 2 and Table 1: the CCL-chemokines (i.e., MCP-1|CCL2 and MIP-1β|CCL4) and CXCL-chemokines (i.e., MIP-2|CXCL2 and KC|CXCL1). The CCL-chemokines are important for stimulating chemotaxis of monocytes out of the bone marrow and in injured tissues to begin the process of repair (28), and CCL4 has an additional role in stimulating migration of natural killer (NK) lymphocytes (28), which are important in surveillance and ultimate clearing of heavily damaged cells (16, 33). CXCL-chemokines primarily trigger release of neutrophils and other immune cells from bone marrow and also function as a chemotactic stimulus for movement of neutrophils in damaged tissues (28). The cytokine G-CSF stimulates granulopoiesis in the bone marrow and works in synergy with MIP-2 and KC to increase several types of circulating leukocytes (68). As importantly in this setting, G-CSF is a critical stimulus for mobilization of adult stem cells from the bone marrow (5). Although IL-6, in combination with its soluble receptor, has been shown to contribute to promotion of progenitor cells (50), its role in this process is not as clearly understood. Some of the chemokines seen in EHS may act like IL-6 and may also have direct protective effects of tissues exposed to stressful conditions, e.g., CXCL1 (3) and G-CSF (36). IL-6 does have extensive effects on immune cell trafficking that include transition from innate to acquired immunity (34) and stimulation of lymphocyte movement across the endothelium and in tissues (17).

The marked elevation in circulating G-CSF is consistent with human data during short-term hyperthermia (41.8°C) where circulating G-CSF rapidly increases in the circulation (54). It is also very modestly increased during exercise in some studies (71) or not at all in others (70), although there may be a closer association with muscle damage than there is with exercise (70). The source of G-CSF in this setting is not known, but muscle fibers have been shown to be capable of secreting G-CSF following lipopolysaccharide exposure (70).

In summary, we have demonstrated that EHS displays a unique pattern of circulating cytokines and cytokine gene expression in muscle that is unlike that seen in PHS, sepsis, or intense exercise. This response is characterized by the greatest elevations in IL-6, and several chemokines, at the beginning of the recovery period. We verified that this pattern of expression is not simply a result of exposure to lower peak T_c or exposure

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to decreased thermal loads but, by elimination, appears to be an effect arising from acute exercise superimposed on heat.

Clinical and Integrative Perspectives

It is apparent from these data that exercise, whether acute or chronic, can play a unique role in the overall immune responsiveness to severe hyperthermia exposure. The data are consistent with the existence of an exercise- and hyperthermia-induced rapid physiological response system that is geared toward initiating survival pathways and recruitment of immune cells involved in rapid wound healing and repair from thermal injury. One would expect that different exercise intensities, levels of exercise training, and the timing of exposure of exertion vs. hyperthermia would likely impact the background immune responsiveness and clinical outcomes in conditions in which EHS can occur.

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DISCLOSURES

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AUTHOR CONTRIBUTIONS

M.A.K. and D.A.M. performed experiments; M.A.K. and T.L.C. analyzed data; M.A.K., L.R.L., and T.L.C. interpreted results of experiments; M.A.K. and T.L.C. prepared figures; M.A.K. drafted manuscript; M.A.K., L.R.L., D.A.M., and T.L.C. edited and revised manuscript; M.A.K., L.R.L., D.A.M., and T.L.C. approved final version of manuscript.

REFERENCES

1. **Allen DL, Harrison BC, Maass A, Bell ML, Byrnes WC, Leinwand LA.** Cardiac and skeletal muscle adaptations to voluntary wheel running in the mouse. *J Appl Physiol (1985)* 90: 1900–1908, 2001.
2. **Alten JA, Moran A, Tsimelzon AI, Mastrangelo M-AA, Hilsenbeck SG, Poli V, Tweardy DJ.** Prevention of hypovolemic circulatory collapse by IL-6 activated Stat3. *PLoS One* 3: e1605, 2008. doi:10.1371/journal.pone.0001605.
3. **Bachmaier K, Toya S, Malik AB.** Therapeutic administration of the chemokine CXCL1/KC abrogates autoimmune inflammatory heart disease. *PLoS One* 9: e89647, 2014. doi:10.1371/journal.pone.0089647.
4. **Barton BE, Jackson JV.** Protective role of interleukin 6 in the lipopolysaccharide-galactosamine septic shock model. *Infect Immun* 61: 1496–1499, 1993.
5. **Bendall LJ, Bradstock KF.** G-CSF: From granulopoietic stimulant to bone marrow stem cell mobilizing agent. *Cytokine Growth Factor Rev* 25: 355–367, 2014. doi:10.1016/j.cytogfr.2014.07.011.
6. **Benjamini Y, Hochberg Y.** Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J R Stat Soc B Methodolog* 57: 289-300, 1995.
7. **Binkley HM, Beckett J, Casa DJ, Kleiner DM, Plummer PE.** National Athletic Trainers' Association Position Statement: Exertional Heat Illnesses. *J Athl Train* 37: 329–343, 2002.
8. **Bouchama A, Bridey F, Hammami MM, Lacombe C, al-Shail E, al-Ohali Y, Combe F, al-Sedairy S, de Prost D.** Activation of coagulation and fibrinolysis in heatstroke. *Thromb Haemost* 76: 909–915, 1996.
9. **Bouchama A, Hammami MM, Al Shail E, De Vol E.** Differential effects of in vitro and in vivo hyperthermia on the production of interleukin-10. *Intensive Care Med* 26: 1646–1651, 2000. doi:10.1007/s001340000665.
10. **Bouchama A, Ollivier V, Roberts G, Al Mohanna F, de Prost D, Eldali A, Saussereau E, El-Sayed R, Chollet-Martin S.** Experimental heatstroke in baboon: analysis of the systemic inflammatory response. *Shock* 24: 332–335, 2005. doi:10.1097/01.shk.0000180620.44435.9c.
11. **Bouchama A, Parhar RS, el-Yazigi A, Sheth K, al-Sedairy S.** Endotoxemia and release of tumor necrosis factor and interleukin 1 alpha in acute heatstroke. *J Appl Physiol (1985)* 70: 2640–2644, 1991.
12. **Bouchama A, Roberts G, Al Mohanna F, El-Sayed R, Lach B, Chollet-Martin S, Ollivier V, Al Baradei R, Loualich A, Nakeeb S, Eldali A, de Prost D.** Inflammatory, hemostatic, and clinical changes in a baboon experimental model for heatstroke. *J Appl Physiol (1985)* 98: 697–705, 2005. doi:10.1152/japplphysiol.00461.2004.
13. **Brattsand R, Linden M.** Cytokine modulation by glucocorticoids: mechanisms and actions in cellular studies. *Aliment Pharmacol Ther* 10, *Suppl* 2: 81–90, 1996.
14. **Brown CO, Salem K, Wagner BA, Bera S, Singh N, Tiwari A, Choudhury A, Buettner GR, Goel A.** Interleukin-6 counteracts therapy-induced cellular oxidative stress in multiple myeloma by up-regulating manganese superoxide dismutase. *Biochem J* 444: 515–527, 2012. doi:10.1042/BJ20112019.
15. **Castell JV, Gómez-Lechón MJ, David M, Fabra R, Trullenque R, Heinrich PC.** Acute-phase response of human hepatocytes: regulation of acute-phase protein synthesis by interleukin-6. *Hepatology* 12: 1179–1186, 1990. doi:10.1002/hep.1840120517.
16. **Chan CJ, Smyth MJ, Martinet L.** Molecular mechanisms of natural killer cell activation in response to cellular stress. *Cell Death Differ* 21: 5–14, 2014. doi:10.1038/cdd.2013.26.
17. **Chen Q, Fisher DT, Clancy KA, Gauguet J-MM, Wang W-C, Unger E, Rose-John S, von Andrian UH, Baumann H, Evans SS.** Fever-range thermal stress promotes lymphocyte trafficking across high endothelial venules via an interleukin 6 trans-signaling mechanism. *Nat Immunol* 7: 1299–1308, 2006. doi:10.1038/ni1406.
18. **Chen T, Guo J, Yang M, Zhu X, Cao X.** Chemokine-containing exosomes are released from heat-stressed tumor cells via lipid raft-dependent pathway and act as efficient tumor vaccine. *J Immunol* 186: 2219–2228, 2011. doi:10.4049/jimmunol.1002991.
19. **Clark AR.** Anti-inflammatory functions of glucocorticoid-induced genes. *Mol Cell Endocrinol* 275: 79–97, 2007. doi:10.1016/j.mce.2007.04.013.
20. **Cressman DE, Greenbaum LE, DeAngelis RA, Ciliberto G, Furth EE, Poli V, Taub R.** Liver failure and defective hepatocyte regeneration in interleukin-6-deficient mice. *Science* 274: 1379–1383, 1996. doi:10.1126/science.274.5291.1379.
21. **Cuzzocrea S, Mazzon E, Dugo L, Centorrino T, Ciccolo A, McDonald MC, de Sarro A, Caputi AP, Thiemermann C.** Absence of endogenous interleukin-6 enhances the inflammatory response during acute pancreatitis induced by cerulein in mice. *Cytokine* 18: 274–285, 2002. doi:10.1006/cyto.2002.0883.
22. **Dawn B, Xuan Y-T, Guo Y, Rezazadeh A, Stein AB, Hunt G, Wu W-J, Tan W, Bolli R.** IL-6 plays an obligatory role in late preconditioning via JAK-STAT signaling and upregulation of iNOS and COX-2. *Cardiovasc Res* 64: 61–71, 2004. doi:10.1016/j.cardiores.2004.05.011.
23. **Dematte JE, O'Mara K, Buescher J, Whitney CG, Forsythe S, McNamee T, Adiga RB, Ndukwu IM.** Near-fatal heat stroke during the 1995 heat wave in Chicago. *Ann Intern Med* 129: 173–181, 1998. doi:10.7326/0003-4819-129-3-199808010-00001.
24. **Deng B, Wehling-Henricks M, Villalta SA, Wang Y, Tidball JG.** IL-10 triggers changes in macrophage phenotype that promote muscle growth and regeneration. *J Immunol* 189: 3669–3680, 2012. doi:10.4049/jimmunol.1103180.
25. **Gleeson M, Bishop NC, Stensel DJ, Lindley MR, Mastana SS, Nimmo MA.** The anti-inflammatory effects of exercise: mechanisms and implications for the prevention and treatment of disease. *Nat Rev Immunol* 11: 607–615, 2011. doi:10.1038/nri3041.
26. **Gong S, Miao Y-L, Jiao G-Z, Sun M-J, Li H, Lin J, Luo M-J, Tan J-H.** Dynamics and correlation of serum cortisol and corticosterone under different physiological or stressful conditions in mice. *PLoS One* 10: e0117503, 2015. doi:10.1371/journal.pone.0117503.
27. **Graber CD, Reinhold RB, Breman JG, Harley RA, Hennigar GR.** Fatal heat stroke. Circulating endotoxin and gram-negative sepsis as complications. *JAMA* 216: 1195–1196, 1971. doi:10.1001/jama.1971.03180330069018.
28. **Griffith JW, Sokol CL, Luster AD.** Chemokines and chemokine receptors: positioning cells for host defense and immunity. *Annu Rev Immunol* 32: 659–702, 2014. doi:10.1146/annurev-immunol-032713-120145.

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29. **Hall DM, Buettner GR, Oberley LW, Xu L, Matthes RD, Gisolfi CV.** Mechanisms of circulatory and intestinal barrier dysfunction during whole body hyperthermia. *Am J Physiol Heart Circ Physiol* 280: H509–H521, 2001.
30. **Hammami MM, Bouchama A, Al-Sedairy S, Shail E, AlOhaly Y, Mohamed GED.** Concentrations of soluble tumor necrosis factor and interleukin-6 receptors in heatstroke and heatstress. *Crit Care Med* 25: 1314–1319, 1997. doi:10.1097/00003246-199708000-00017.
31. **Helwig BG, Leon LR.** Tissue and circulating expression of IL-1 family members following heat stroke. *Physiol Genomics* 43: 1096–1104, 2011. doi:10.1152/physiolgenomics.00076.2011.
32. **Hubbard RW, Bowers WD, Matthew WT, Curtis FC, Criss RE, Sheldon GM, Ratteree JW.** Rat model of acute heatstroke mortality. *J Appl Physiol Respir Environ Exerc Physiol* 42: 809–816, 1977.
33. **Iannello A, Raulet DH.** Immune surveillance of unhealthy cells by natural killer cells. *Cold Spring Harb Symp Quant Biol* 78: 249–257, 2013. doi:10.1101/sqb.2013.78.020255.
34. **Jones SA.** Directing transition from innate to acquired immunity: defining a role for IL-6. *J Immunol* 175: 3463–3468, 2005. doi:10.4049/jimmunol.175.6.3463.
35. **King MA, Leon LR, Mustico DL, Haines JM, Clanton TL.** Biomarkers of multiorgan injury in a preclinical model of exertional heat stroke. *J Appl Physiol (1985)* 118: 1207–1220, 2015. doi:10.1152/japplphysiol.01051.2014.
36. **Kojima H, Otani A, Oishi A, Makiyama Y, Nakagawa S, Yoshimura N.** Granulocyte colony-stimulating factor attenuates oxidative stress-induced apoptosis in vascular endothelial cells and exhibits functional and morphologic protective effect in oxygen-induced retinopathy. *Blood* 117: 1091–1100, 2011. doi:10.1182/blood-2010-05-286963.
37. **Lauritzen HPMM, Brandauer J, Schjerling P, Koh H-J, Treebak JT, Hirshman MF, Galbo H, Goodyear LJ.** Contraction and AICAR stimulate IL-6 vesicle depletion from skeletal muscle fibers in vivo. *Diabetes* 62: 3081–3092, 2013. doi:10.2337/db12-1261.
38. **Leon LR.** Heat stroke and cytokines. *Prog Brain Res* 162: 481–524, 2007. doi:10.1016/S0079-6123(06)62024-4.
39. **Leon LR, Blaha MD, DuBose DA.** Time course of cytokine, corticosterone, and tissue injury responses in mice during heat strain recovery. *J Appl Physiol (1985)* 100: 1400–1409, 2006. doi:10.1152/japplphysiol.01040.2005.
40. **Leon LR, DuBose DA, Mason CW.** Heat stress induces a biphasic thermoregulatory response in mice. *Am J Physiol Regul Integr Comp Physiol* 288: R197–R204, 2005. doi:10.1152/ajpregu.00046.2004.
41. **Leon LR, White AA, Kluger MJ.** Role of IL-6 and TNF in thermoregulation and survival during sepsis in mice. *Am J Physiol* 275: R269–R277, 1998.
42. **Li Y, Xing W, He Y-Z, Chen S, Rhodes SD, Yuan J, Zhou Y, Shi J, Bai J, Zhang F-K, Yuan W-P, Cheng T, Xu M-J, Yang F-C.** Interleukin 8/KC enhances G-CSF induced hematopoietic stem/progenitor cell mobilization in Fancg deficient mice. *Stem Cell Investig* 1: 19, 2014. http://sci.amegroups.com/article/view/4965.
43. **Malamud N, Haymaker W, Custer RP.** Heat stroke; a clinico-pathologic study of 125 fatal cases. *Mil Surg* 99: 397–449, 1946.
44. **Matsumoto T, O'Malley K, Efron PA, Burger C, McAuliffe PF, Scumpia PO, Uchida T, Tschoeke SK, Fujita S, Moldawer LL, Hemming AW, Foley DP.** Interleukin-6 and STAT3 protect the liver from hepatic ischemia and reperfusion injury during ischemic preconditioning. *Surgery* 140: 793–802, 2006. doi:10.1016/j.surg.2006.04.010.
45. **Navarro F, Bacurau AVN, Pereira GB, Araújo RC, Almeida SS, Moraes MR, Uchida MC, Costa Rosa LFBP, Navalta J, Prestes J, Bacurau RFP.** Moderate exercise increases the metabolism and immune function of lymphocytes in rats. *Eur J Appl Physiol* 113: 1343–1352, 2013. doi:10.1007/s00421-012-2554-y.
46. **Ostrowski K, Rohde T, Asp S, Schjerling P, Pedersen BK.** Pro- and anti-inflammatory cytokine balance in strenuous exercise in humans. *J Physiol* 515: 287–291, 1999. doi:10.1111/j.1469-7793.1999.287ad.x.
47. **Ostrowski K, Rohde T, Zacho M, Asp S, Pedersen BK.** Evidence that interleukin-6 is produced in human skeletal muscle during prolonged running. *J Physiol* 508: 949–953, 1998. doi:10.1111/j.1469-7793.1998.949bp.x.
48. **Peake JM, Della Gatta P, Suzuki K, Nieman DC.** Cytokine expression and secretion by skeletal muscle cells: regulatory mechanisms and exercise effects. *Exerc Immunol Rev* 21: 8–25, 2015.
49. **Pedersen BK, Febbraio MA.** Muscle as an endocrine organ: focus on muscle-derived interleukin-6. *Physiol Rev* 88: 1379–1406, 2008. doi:10.1152/physrev.90100.2007.
50. **Peters M, Müller AM, Rose-John S.** Interleukin-6 and soluble interleukin-6 receptor: direct stimulation of gp130 and hematopoiesis. *Blood* 92: 3495–3504, 1998.
51. **Phillips NA, Welc SS, Wallet SM, King MA, Clanton TL.** Protection of intestinal injury during heat stroke in mice by interleukin-6 pretreatment. *J Physiol* 593: 739–752, 2015. doi:10.1113/jphysiol.2014.283416.
52. **Rhind SG, Gannon GA, Shephard RJ, Buguet A, Shek PN, Radomski MW.** Cytokine induction during exertional hyperthermia is abolished by core temperature clamping: neuroendocrine regulatory mechanisms. *Int J Hyperthermia* 20: 503–516, 2004. doi:10.1080/02656730410001670651.
53. **Roberts GT, Ghebeh H, Chishti MA, Al-Mohanna F, El-Sayed R, Al-Mohanna F, Bouchama A.** Microvascular injury, thrombosis, inflammation, and apoptosis in the pathogenesis of heatstroke: a study in baboon model. *Arterioscler Thromb Vasc Biol* 28: 1130–1136, 2008. doi:10.1161/ATVBAHA.107.158709.
54. **Robins HI, Kutz M, Wiedemann GJ, Katschinski DM, Paul D, Grosen E, Tiggelaar CL, Spriggs D, Gillis W, d'Oleire F.** Cytokine induction by 41.8 degrees C whole body hyperthermia. *Cancer Lett* 97: 195–201, 1995. doi:10.1016/0304-3835(95)03976-4.
55. **Sakata H, Narasimhan P, Niizuma K, Maier CM, Wakai T, Chan PH.** Interleukin 6-preconditioned neural stem cells reduce ischaemic injury in stroke mice. *Brain* 135: 3298–3310, 2012. doi:10.1093/brain/aws259.
56. **Sawka MN, Leon LR, Montain SJ, Sonna LA.** Integrated physiological mechanisms of exercise performance, adaptation, and maladaptation to heat stress. *Compr Physiol* 1: 1883–1928, 2011. doi:10.1002/cphy.c100082.
57. **Steensberg A, Fischer CP, Keller C, Møller K, Pedersen BK.** IL-6 enhances plasma IL-1ra, IL-10, and cortisol in humans. *Am J Physiol Endocrinol Metab* 285: E433–E437, 2003. doi:10.1152/ajpendo.00074.2003.
58. **Steensberg A, van Hall G, Osada T, Sacchetti M, Saltin B, Klarlund Pedersen B.** Production of interleukin-6 in contracting human skeletal muscles can account for the exercise-induced increase in plasma interleukin-6. *J Physiol* 529: 237–242, 2000. doi:10.1111/j.1469-7793.2000.00237.x.
59. **Tidball JG, Villalta SA.** Regulatory interactions between muscle and the immune system during muscle regeneration. *Am J Physiol Regul Integr Comp Physiol* 298: R1173–R1187, 2010. doi:10.1152/ajpregu.00735.2009.
60. **Tilg H, Trehu E, Atkins MB, Dinarello CA, Mier JW.** Interleukin-6 (IL-6) as an anti-inflammatory cytokine: induction of circulating IL-1 receptor antagonist and soluble tumor necrosis factor receptor p55. *Blood* 83: 113–118, 1994.
61. **Toft AD, Jensen LB, Bruunsgaard H, Ibfelt T, Halkjaer-Kristensen J, Febbraio M, Pedersen BK.** Cytokine response to eccentric exercise in young and elderly humans. *Am J Physiol Cell Physiol* 283: C289–C295, 2002. doi:10.1152/ajpcell.00583.2001.
62. **Wallace RF, Kriebel D, Punnett L, Wegman DH, Amoroso PJ.** Prior heat illness hospitalization and risk of early death. *Environ Res* 104: 290–295, 2007. doi:10.1016/j.envres.2007.01.003.
63. **Welc SS, Clanton TL.** The regulation of interleukin-6 implicates skeletal muscle as an integrative stress sensor and endocrine organ. *Exp Physiol* 98: 359–371, 2013. doi:10.1113/expphysiol.2012.068189.
64. **Welc SS, Clanton TL, Dineen SM, Leon LR.** Heat stroke activates a stress-induced cytokine response in skeletal muscle. *J Appl Physiol (1985)* 115: 1126–1137, 2013. doi:10.1152/japplphysiol.00636.2013.
65. **Welc SS, Judge AR, Clanton TL.** Skeletal muscle interleukin-6 regulation in hyperthermia. *Am J Physiol Cell Physiol* 305: C406–C413, 2013. doi:10.1152/ajpcell.00084.2013.
66. **Welc SS, Morse DA, Mattingly AJ, Laitano O, King MA, Clanton TL.** The Impact of Hyperthermia on Receptor-Mediated Interleukin-6 Regulation in Mouse Skeletal Muscle. *PLoS One* 11: e0148927, 2016. doi:10.1371/journal.pone.0148927.
67. **Welc SS, Phillips NA, Oca-Cossio J, Wallet SM, Chen DL, Clanton TL.** Hyperthermia increases interleukin-6 in mouse skeletal muscle. *Am J Physiol Cell Physiol* 303: C455–C466, 2012. doi:10.1152/ajpcell.00028.2012.
68. **Wengner AM, Pitchford SC, Furze RC, Rankin SM.** The coordinated action of G-CSF and ELR + CXC chemokines in neutrophil mobilization during acute inflammation. *Blood* 111: 42–49, 2008. doi:10.1182/blood-2007-07-099648.

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69. **Werner S, Grose R.** Regulation of wound healing by growth factors and cytokines. *Physiol Rev* 83: 835–870, 2003. doi:10.1152/physrev.00031.2002.
70. **Wright CR, Brown EL, Della Gatta PA, Fatouros IG, Karagounis LG, Terzis G, Mastorakos G, Michailidis Y, Mandalidis D, Spengos K, Chatzinikolaou A, Methenitis S, Draganidis D, Jamurtas AZ, Russell AP.** Regulation of Granulocyte Colony-Stimulating Factor and Its Receptor in Skeletal Muscle is Dependent Upon the Type of Inflammatory Stimulus. *J Interferon Cytokine Res* 35: 710–719, 2015. doi:10.1089/jir.2014.0159.
71. **Yamada M, Suzuki K, Kudo S, Totsuka M, Nakaji S, Sugawara K.** Raised plasma G-CSF and IL-6 after exercise may play a role in neutrophil mobilization into the circulation. *J Appl Physiol (1985)* 92: 1789–1794, 2002. doi:10.1152/japplphysiol.00629.2001.
72. **Yáñez-Mó M, Siljander PR-M, Andreu Z, Zavec AB, Borràs FE, Buzas EI, Buzas K, Casal E, Cappello F, Carvalho J, Colás E, Cordeiro-da Silva A, Fais S, Falcon-Perez JM, Ghobrial IM, Giebel B, Gimona M, Graner M, Gursel I, Gursel M, Heegaard NHH, Hendrix A, Kierulf P, Kokubun K, Kosanovic M, Kralj-Iglic V, Krämer-Albers E-M, Laitinen S, Lässer C, Lener T, Ligeti E, Linē A, Lipps G, Llorente A, Lötvall J, Manček-Keber M, Marcilla A, Mittelbrunn M, Nazarenko I, Nolte-'t Hoen ENM, Nyman TA, O'Driscoll L, Olivan M, Oliveira C, Pállinger É, Del Portillo HA, Reventós J, Rigau M, Rohde E, Sammar M, Sánchez-Madrid F, Santarém N, Schallmoser K, Ostenfeld MS, Stoorvogel W, Stukelj R, Van der Grein SG, Vasconcelos MH, Wauben MHM, De Wever O.** Biological properties of extracellular vesicles and their physiological functions. *J Extracell Vesicles* 4: 27066, 2015. doi:10.3402/jev.v4.27066.

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Major Metabolic Hormone Responses to Exertional Heat Stroke in Mice

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Abstract

Disordered glucose metabolism has been shown to be a strong prognostic indicator of poor outcomes in heat stroke. In mice, within the first few hours of recovery from exertional heat stroke (EHS), glucose is reduced; however, by 24 h and for up to 4 days, sustained hyperglycemia has also been observed. To gain perspective on the underlying mechanisms that contribute to these responses we looked at circulating metabolic hormones responsible for glucose regulation. Male mice (n= 8, per group) ran in forced running wheels within an enclosed climatic chamber at 37.5°C/35% relative humidity (RH) until loss of cognitive function (approx. 2 h). Animals were sacrificed at 0.5h, 3h, 24h, 4d, 9d or 14d post-EHS. Plasma samples were collected and metabolic hormones analyzed using Luminex multiplex technology or ELISA (corticosterone). Hormones secreted by the pancreas, amylin ($p=0.02$), c-peptide ($p=0.003$), and insulin ($p=0.003$) were markedly suppressed at 0.5 h and continued to be suppressed at 3 h. Expected glucagon responses were absent during this period. Cytokines that influence glucose metabolism or uptake, IL-6 and MCP-1, displayed a significant increase at 0.5 and 3 h ($p=0.0004$, $p=0.0008$ respectively), with peak concentrations appearing at 0.5 h. Resistin (secreted by white adipose) was also significantly elevated at 0.5 h ($p=0.003$) and then decreased to low levels at 3h ($p<0.002$). Corticosterone was significantly elevated at the 0.5 h ($p=0.0015$) and 3 h time points ($p=0.0009$). These results may reflect, in part, hypoglycemia seen following exercise in the heat but are also consistent with transient organ dysfunction, specifically in the liver (i.e. failure to elevate glucose) and/or pancreas (suppression of insulin secretion without compensating glucagon response).
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We recommend

Differences in tolerance to exertional hyperthermia between male and female mice
Christian Kyle Garcia et al., FASEB J, 2017

Exertional vs. Passive Heat Stroke: Altered Time Course of Cytokine Expression in Plasma and Skeletal Muscle
Michelle King et al., FASEB J, 2015

Exposure to Oxidized Tyrosine Products Induced Glycometabolism Disorder Involving Thyroid Hormones Resistance in C57BL/6 mice
Yinyi Ding et al., FASEB J, 2017

Role of carotid bodies in glucose counterregulation in type 1 diabetes (709.2)
Simmi Dube et al., FASEB J, 2014

Direct activating effects of adrenocorticotropic hormone (ACTH) on brown adipose tissue are attenuated by corticosterone.
Johanna C van den Beukel et al., FASEB J, 2014

High insulin levels tied to obesity pathway
UT Southwestern Medical Center, ScienceDaily, 2014

ADA Key Takeaway: Use of GLP-1 Receptor Agonist Therapy With Insulin
PracticeUpdate, 2014

Pinpointing Role Of Insulin On Glucagon Levels
Joslin Diabetes Center, ScienceDaily, 2009

Multiple Metabolic Abnormalities Seen in Lupus
MedPage Today, 2017

Pancreatic signals controlling food intake; insulin, glucagon and amylin.
Stephen C Woods et al., Philosophical Transactions B, 2006

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Differences in tolerance to exertional hyperthermia between male and female mice

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Abstract

Surveillance reports suggest possible reductions in heat stroke susceptibility in female vs. male active component members of the US Armed Forces, but whether these differences reflect behavior or underlying biology is unknown. Previous studies in mice have shown that females exhibit markedly better resistance to moderate, passive heat. However, whether heat tolerance translates to acute settings or to exertional heat stroke (EHS) has not been tested. In this study, we compared responses in male and female mice to an established model of EHS. Exercise trained mice (3 wks) were maintained at 37.5°C (35% RH) and ran using a preprogrammed incremental protocol on a forced running wheel. The EHS end point was defined as loss of consciousness. Female mice on average ran longer than males (177 vs. 124 min; p=0.0001), and were exposed to greater heat loads (241 vs. 160 °C · min; p=0.0001). Male and female mice ran to nearly identical average peak core temperatures, both 42.2°C (n.s.). There were no differences in the minimum temperature during post EHS hypothermia 32°C (n.s.) or the time to reach the minimum temperature. However, females lost a greater % body weight (9.2% vs 7.5% $p < 0.001$), demonstrated significantly higher levels of circulating corticosterone (286 vs 183 ng/ml, $p = 0.001$, 3 h) and higher levels of resistin polypeptide (8891 vs. 3781 pg/ml, $p = 0.004$, 3 h). These results demonstrate that female mice have greater resistance to EHS during exercise in hyperthermia. Possible mechanisms include greater body surface to mass ratio in females vs. males (3.3 vs. 3.2 m^2/kg; p=0.0001), greater aerobic conditioning in females

(characteristic of mice), or a hormonally or genetically induced resistance to hyperthermia. Though controversial, marked elevations in circulating corticosterone and resistin in females have the capacity to contribute to improved heat tolerance. We conclude that female mice are significantly more resistant to EHS than male mice. Inherent thermal tolerance in female mice may provide an evolutionary advantage because metabolic rate and heat production have been shown to double during pregnancy and lactation. *Author views not official US Army or DoD policy. W81XWH-15-2-0038*

We recommend

Major Metabolic Hormone Responses to Exertional Heat Stroke in Mice
Gerard Patrick Robinson et al., FASEB J, 2017

Effects of environmental temperature and humidity on a model of exertional heat stroke in mice (1104.26)
Michelle King et al., FASEB J, 2014

Hysteresis in the heart rate-core temperature relationship during acute heat stress in rats: implications for systemic hemodynamics
Nisha Charkoudian et al., FASEB J, 2012

Postexercise thermal and cardiovascular responses following fast and slow heating exercise protocols
Glen P Kenny et al., FASEB J, 2011

Ibuprofen Pre-Treatment Increases Biomarkers of Heat Stroke Severity and Attenuates Fever during Recovery in Conscious Rats
Gerald N Audet et al., FASEB J, 2016

Moderate stress protects female mice against bacterial infection of the bladder by eliciting uroepithelial shedding.
E Dalal, Infect Immun, 1994

Whole-body heat stress lowers exercise capacity, blood flow in men
American Physiological Society (APS), ScienceDaily, 2017

Oral Presentations 2016 AANS Annual Scientific Meeting Chicago, IL • April 30–May 4, 2016Published online April 1, 2016; DOI: 10.3171/2016.4.JNS.AANS2016abstracts
J Neurosurg, 2016

Hemodynamic Basis of Exercise Tolerance in Tricuspid Regurgitation
PracticeUpdate, 2014

Moist-Heat Resistance, Spore Aging, and Superdormancy in Clostridium difficile ▿ †
Alexander Rodriguez-Palacios, Appl Environ Microbiol, 2010

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